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A CYTOCHEMICAL STUDY OF THE RESPONSES OF THE ADRENAL CORTEX OF THE RAT TO THIAMINE, RIBOFLAVIN, AND PYRIDOXINE DEFICIENCIES [1,2]

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FOURTEEN FIGURES

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The current concept of adrenal physiology is that at least 2 types of hormones are elaborated in the adrenal cortex. The first of these, which may be described in terms of its prototype desoxycorticosterone, regulates the concentration of electrolytes in the tissues and is apparently elaborated in the glomerular zone of the adrenal cortex. The second, the prototype of which is corticosterone, controls gluconeogenesis and appears to be secreted by the cells of the zona fasciculata. The first of these hormones does not seem to be regulated importantly by the anterior pituitary gland, whereas the second is (Swann, '40; Deane and Greep, '46). Therefore, the observation that pantothenic acid deficiency caused enlargement of the adrenal gland, accompanied by a reduction in the steroid content of the fasciculata and by a decrease in weight of the thymus (an index of pituitary-adrenal activity), suggests that an effect of the vitamin deficiency had been to stimulate an

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[2] We are indebted to Merck and Company, Inc., Rahway, N. J., for the crystalline vitamins used in these studies.

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excessive secretion of corticosterone and its homologues (Deane and McKibbin, '46). Such a response is entirely comparable to the production of the adaptation syndrome of Selye ('46), in which physiological stress leads to a homeostatic increase in the activity of the pituitary and adrenal glands.

Since pantothenic acid deficiency thus alters the activity of the biological mechanism which regulates gluconeogenesis, it seemed possible that this alteration might be a reaction to a disturbance in carbohydrate metabolism. Therefore other disturbances in carbohydrate metabolism might cause the same effects. To test this possibility, deficiencies in thiamine and riboflavin, known to be constituents of coenzymes for the oxidation of carbohydrates, were studied as well as a deficiency of pyridoxine, believed to regulate transamination (Hawkins, MacFarland and McHenry, '46). After the first experiment had shown that thiamine deficiency rapidly activated the pituitary-adrenal-thymus system, the study was extended to determine whether the response was caused by the vitamin deficiency *per se* or by the partial inanition induced by the deficiency.

MATERIAL AND METHODS

For the first experiment, 20 young male rats of the Long-Evans strain were used. Seven controls were fed a purified diet (table 1) essentially similar to that employed by Deane and McKibbin, but with the addition of *p*-aminobenzoic acid, inositol and biotin in an attempt to prevent the fatty infiltration of the liver observed in the control animals of the previous investigation. Five rats were maintained on this purified ration lacking thiamine, 4 on the ration lacking riboflavin, and 4 on the ration lacking pyridoxine. Control and deficient animals were killed together at intervals according to the severity of the deficiency signs (table 2). The rats were killed in the forenoon, when glycogen is normally present in the liver and its quantity may be judged.

For the second experiment, 20 young male rats of the Long-Evans strain were distributed in groups of 4 according

to weight. Of each group, 1 was fed the thiamine deficient diet (TD) and the other 3 were fed the control purified diet. One of the controls was paired with the deficient rat so that its weight was similarly restricted (WR), and the second had its food intake restricted (IR) to that of the deficient. The third was permitted to eat *ad libitum* (AL). Groups were killed at the end of 1, 2 and 3 weeks.

TABLE 1

Constituents of complete purified diet.

BASAL MIXTURE		VITAMINS AND OTHER SUPPLEMENTS			
Sucrose (gm)	67	Thiamine-HCl (μg)	250		
Casein, purified [1] (gm)	24	Pyridoxine-HCl (μg)	250	Choline chloride (mg)	100
Salt mixture [2] (gm)	4	Riboflavin (μg)	300	Inositol (mg)	100
Corn oil (gm)	5	Niacin (mg)	2.5	Irradiated ergosterol (I.U.)	310
		Ca-pantothenate (mg)	2	α-tocopherol (mg)	5
		p-aminobenzoic acid (mg)	30	β-carotene (mg)	1.1
		Biotin (μg)	40	2-methyl,1,4-naphthoquinone (μg)	600

[1] SMA.

[2] Phillips and Hart.

At autopsy the paired adrenals and the thymus of each animal were weighed on a Roller-Smith precision balance. The adrenals and pieces of liver were then fixed in 10% neutralized formalin for lipids and in Maximow's Zenker-formalin for mitochondria. In addition, in the first experiment, pieces of liver were fixed for glycogen in Rossman's picro-alcohol-formalin.

For the cytochemical demonstration of lipids, the formalin-fixed blocks were cut on a freezing microtome at 15 μ. From each block 1 section was stained with sudan IV and Harris' hematoxylin, another with sudan black B, and a third by the

Schiff plasmal method. A fourth section was mounted unstained, and a fifth was extracted with acetone before mounting. The latter sections were viewed on the polarizing and fluorescence microscopes for substances which are birefringent, possess a greenish-white autofluorescence, and are acetone-soluble. Any acetone-soluble, sudanophilic material which is also Schiff-positive, birefringent and autofluorescent may be considered a ketosteroid, since no other single class of substances gives all of these reactions (Dempsey and Wislocki, '46).

For mitochondrial preparations the Maximov-fixed blocks were postchromated for 3 days at 37°C., sectioned in paraffin at 3 μ, and stained with Mallory's phosphotungstic acid hematoxylin. To demonstrate liver glycogen, the pieces fixed in Rossman's fluid were sectioned in paraffin at 5 μ and stained by the Bauer-Feulgen technique (Bensley, '39).

OBSERVATIONS

Deficiencies of thiamine, riboflavin, and pyridoxine

Weight changes. Table 2 presents the changes in the weight of the rats and the proportional weights of their thymuses and paired adrenals in the first experiments. The controls grew steadily and well on the purified diet. The thiamine deficient rats ceased to gain weight by the end of the second week, were losing weight by the end of the third, and were below their initial weight and moribund after 4 weeks on the diet. The rats deficient in riboflavin and in pyridoxine continued to gain weight at a slow rate throughout the experimental period of 10 weeks, never becoming moribund. The only period of marked illness in these 2 groups occurred between the second and fourth weeks, after which there was some regression of the acuteness of the signs of their deficiencies.

In the control group, the weights of the paired adrenals and of the thymus gland drop gradually in relation to body weight, as is characteristic of normal ageing (Korenchevsky, '42).

In thiamine deficiency, on the other hand, a progressive increase in the relative weight of the adrenals occurs during the experimental period of 4½ weeks, and the atrophy of the thymus is far more rapid than normal. In the absence of

TABLE 2

Data on rats fed diets deficient in thiamine, or riboflavin, or pyridoxine and their controls.

DIET	ANIMAL	WEEKS ON DIET	INITIAL WT.	PEAK WT.[1]	TERM WT.	WT. CHANGE	ADRENAL PROP.[2]	THYMUS PROP.[2]
			gm	*gm*	*gm*	*gm*	*mg/100 gm*	
Control	A	3	62	140 (21d)	140	+ 78	16.2	260
	B	3	56	138 (21d)	138	+ 82	21.3	402
	C	4	56	166 (28d)	166	+ 110	23.5	332
	D	6	56	198 (42d)	198	+ 142	14.5	234
	E	8	74	258 (56d)	258	+ 184	15.1	236
	F	10	70	241 (70d)	241	+ 141	13.5	155
	G	10	49	229 (70d)	229	+ 180	14.2	92
Thiamine deficient	A	3	92	130 (14d)	112	+ 10	27.1	61
	B	3	94	135 (14d)				
	C	3	72	102 (14d)	72	0	29.8	124
	D	4	97	143 (14d)	93	— 4	38.6	27
	E	4½	102	139 (14d)	94	— 8	38.4	62
Riboflavin deficient	A	3	57	77 (14d)	65	+ 8	30.9	169
	B	6	57	76 (42d)	76	+ 19	26.0	47
	C	8	73	99 (56d)	99	+ 26	18.8	117
	D	10	73	133 (63d)	132	+ 59	16.8	148
Pyridoxine deficient	A	3	42	67 (21d)	67	+ 25	25.8	51
	B	6	44	83 (24d)	70	+ 26	32.6	42
	C	8	46	114 (56d)	114	+ 68	22.8	
	D	10	42	123 (70d)	123	+ 81	18.2	86

[1] Data in parentheses give day when their maximum weight was attained.
[2] Proportional weight.

riboflavin or pyridoxine from the diet, however, the adrenals remain relatively large up to 6 weeks and then become smaller. Riboflavin deficiency causes little change in the thymus gland, whereas pyridoxine deficiency results in considerable atrophy. Thus the 3 deficiencies studied produce entirely different patterns of response in the adrenals and thymus.

Histological results. a. Controls rats. In all controls, the adrenal cortex presents the normal picture. Four zones are distinguishable by the organization of the cells and by their lipid contents. The subcapsular zone, or glomerulosa, is composed of loops of cells filled with moderate sized, deeply staining sudanophilic droplets (fig. 1) which are, in addition, acetone soluble, Schiff-positive (fig. 4), autofluorescent, and birefringent (fig. 8). Here the birefringent particles are crowded and usually both coarse and fine in size. (The work of Weaver and Nelson ('43) indicates that small particles are in the process of being released from the cells, whereas the coarse ones are storage material.) There follows a narrow, fat-free transitional zone. Then comes the zona fasciculata, comprising long cords of cells separated by narrow sinusoids. On the basis of lipid content this zone may be divided into 2 sections — a broad, outer region in which the cells are swollen with small, pale sudanophilic droplets and a narrower, inner region in which lipids are greatly reduced in amount. The lipid droplets of the fasciculata contain ketosteroids as evidenced by the battery of histochemical tests. The birefringent material is sparser here than in the glomerulosa, especially in the younger animals, and consists of scattered coarse particles with many fine ones intermixed. The cords of cells are somewhat disorganized immediately adjacent to the medulla (zona reticularis), and the sinusoids anastomose into larger vessels. Here the cells contain sudanophilic droplets of irregular size which fail to display all of the ketosteroid reactions and probably may be considered to be triglycerides.

Mitochondrial preparations of the adrenals of these rats likewise present the normal picture. These organelles are fine granules in the glomerulosa, slightly larger spheres in the outer fasciculata, and again small granules in the inner fasciculata and reticularis (Deane and Greep, '46, figs. 22 and 23).

The livers of some of these rats exhibit some fatty infiltration but far less extremely than with the previous diet (Deane and McKibbin). Very little infiltration occurs by 6 weeks, but a moderate accumulation is variably present by 10 weeks. The

fat occurs in small droplets in the central cells of the lobule, but it fails to present the ketosteroid reactions observed in the former investigation. Apparently the addition of more vitamins to the purified diet partially rectifies the conditions leading to a fatty liver. This may be due principally to the inositol, which is known to possess lipotropic activity (Best et al., '46). A moderate amount of glycogen occurs in these livers and appears mostly in the peripheral part of the lobule. The hepatic cell mitochondria are small and exhibit the usual zonation by being spherical in the cells at the periphery of the lobule, rod-shaped in the intermediate zone, and fibrillar and very light staining in the center.

b. Thiamine deficient rats. The dietary lack of thiamine produces essentially the same alterations in the adrenal cortex of the rat that were observed in pantothenic acid deficiency. At 3 weeks the adrenal sections stained with sudans show an unchanged zona glomerulosa but a fasciculata containing less lipid than in the control preparations. Moreover, the fat-free transitional zone has disappeared. The Schiff reaction is intense in the glomerulosa but very faint in the fasciculata, where it is limited to the outer-most layers. A normal quantity of birefringent particles occurs in the glomerulosa, whereas in the fasciculata the birefringence is increased beyond the normal amount. Autofluorescence of usual intensity persists in the glomerulosal cells and in the outer half of the fasciculata.

At 4 and 4½ weeks, although the lipid of the glomerulosa is of almost normal quantity and retains all of the ketosteroid characteristics, sudanophilia has virtually disappeared from the fasciculata (fig. 3). Furthermore, the small amount of residual lipid does not possess the ketosteroid reactions.

Whereas the mitochondria in the cells of the zona glomerulosa in these adrenals are the usual small granules, those in the fasciculata differ distinctly from normal. They are greatly swollen, irregular in size within individual cells, and stain very slightly.

The liver preparations for these animals exhibit the following characteristics. Sudanophilic lipids are completely absent.

Glycogen is present in extremely small amounts. The phosphotungstic-acid hematoxylin preparations reveal that the hepatic cells are smaller than normal and the mitochondria are swollen, vesiculated and irregular in size.

c. Riboflavin deficient rats. The rats lacking riboflavin in the diet were killed at 3, 6, 8 and 10 weeks, respectively. The concentration and distribution of sudanophil lipids in the adrenals are within the usual range for all 4, and the ketosteroid tests are essentially normal, although the transitional zone has been obliterated (fig. 5). The quantity of birefringent particles is somewhat increased in the outer fasciculata. Paraffin sections of the adrenals, moreover, reveal that the fasciculata cells are smaller than normal but the mitochondria are spheres of the usual size.

The livers of the riboflavin deficient rats display a fatty infiltration at 3 and 6 weeks which is considerably greater than that which occurs in the corresponding control rats. Moreover, the fat is concentrated in the peripheral cells of the lobule and occurs in large-sized drops. It fails to give the ketosteroid reactions. By 8 and 10 weeks of deficiency, this fatty infiltration has disappeared. Glycogen is moderately concentrated in the liver cells and occurs chiefly near the central vein of the lobule. The mitochondria are normal.

d. Pyridoxine deficient rats. The 4 rats fed the pyridoxine deficient diet were killed at 3, 6, 8, and 10 weeks, respectively. Cytochemically their adrenal cortices indicate some stimulation at 3 weeks which disappears later. Thus at 3 weeks, although the glomerulosa appears normal, the fasciculata and reticularis display an augmented amount of sudanophilic lipids which fill all of the cells to the border of the medulla. This lipid is Schiff-positive and moderately autofluorescent, and the whole region contains crowded fine birefringent particles. At 6 and 10 weeks, however, the appearance of the lipid preparations is no different from that of the controls (fig. 6). The mitochondria are fine granules of normal appearance in all these adrenals.

The livers of the pyridoxine deficient rats are uniformly free of histologically demonstrable fat. They exhibit a moderate concentration of glycogen which is greater at the periphery of the lobule than at the center. The mitochondria are entirely normal.

Comparison of reactions to thiamine deficiency and to inanition

Weight changes. Five groups of 4 young male rats each were compared for the effect on the adrenal cortex and thymus of thiamine deficiency and comparable degree of inanition. Inspection of table 3 reveals that the deficient rats (TD) ceased gaining weight on about the eleventh day, the weight restricted (WR) rats on the twelfth, and the intake restricted rats (IR) about a day later. The *ad libitum* controls (AL) gained steadily and well. The adrenals of the deficient rats weigh proportionately most and their thymuses least; the weight restricted and intake restricted rats have large adrenals but larger thymuses; and the controls have the smallest adrenals and the largest thymuses. At 3 weeks the response seems to be most marked for group C, which comprised the youngest and smallest rats.

Histological results. a. Control rats. The 5 control rats (AL) all display normal adrenal cortices such as have been described above (figs. 7 and 8). Their livers contain only occasional fatty cells and present no other histological abnormalities.

b. Thiamine deficient rats. The rats fed the thiamine deficient diet (TD) show a progressive alteration of the adrenal cortex during the 3 weeks experimental period. The sequence of changes is unfortunately complicated by the fact that the larger animals are more reistant to the deficiency than are the smaller ones. Nevertheless, it appears to be as follows. By the end of 1 week (A) there is a slight reduction of sudanophil lipids in the fasciculata (fig. 2). By the end of 2 weeks (B), pituitary stimulation of the cortex is indicated not only by the increased weight of the adrenal and atrophy

of the thymus, but also by the augmentation of lipids in the fasciculata. Sudanophilic and birefringent material is increased in quantity and extends down to the border of the medulla (as in fig. 10). By 3 weeks the lipid content of the fasciculata cells is again reduced, more so in the smallest rat (C) than in the other 2 (D and E). In all 3 adrenals, lipids

TABLE 3

Data on thiamine deficient rats and their controls.

EXP.	GROUP	WEEKS	INITIAL WT.	PEAK WT.[1]	TERM. WT.	WT. CHANGE	ADRENAL PROP.[2]	THYMUS PROP.[2]
			gm	*gm*	*gm*	*gm*	*mg/100 gm*	
Thiamine	A	1	60	88 (7d)	88	+ 28	21.2	336
deficient	B	2	75	99 (11d)	95	+ 20	25.0	192
(TD)	C	3	48	82 (9d)	60	+ 12	35.0	68
	D	3	57	86 (11d)	71	+ 14	26.6	100
	E[3]	3	82	117 (11d)	86	+ 4	26.5	44
			64.4	94.4	80	+ 15.6	26.9	148
Controls	A	1	68	94 (7d)	94	+ 26	18.9	330
restricted	B	2	77	108 (7d)	105	+ 28	22.3	275
in weight	C	3	53	90 (12d)	70	+ 17	28.4	73
(WR)	D	3	61	94 (12d)	76	+ 15	28.9	86
	E	3	85	129 (12d)	103	+ 18	26.1	159
			68.8	103	89.6	+ 20.8	25.1	185
Controls	A	1	63	88 (7d)	88	+ 25	22.2	414
restricted	B	2	74	105 (9d)	102	+ 28	26.0	302
in food	C	3	47	94 (14d)	66	+ 19	30.9	71
intake	D	3	51	87 (12d)	79	+ 28	27.0	215
(IR)	E	3	85	150 (14d)	102	+ 18	26.0	302
			64	104.8	87.4	+ 23.4	26.5	261
Controls	A	1	65	89 (7d)	89	+ 24	19.8	279
fed	B	2	69	135 (14d)	135	+ 66	17.1	336
ad libitum	C	3	51	152 (21d)	152	+ 101	15.4	270
(AL)	D	3	65	155 (21d)	155	+ 90	16.7	406
	E	3	82	141 (21d)	141	+ 59	17.2	184
			66.4	134.4	134.4	+ 68	17.2	295

[1] Data in parentheses indicate day when maximum weight was attained.
[2] Proportional weight.
[3] Wasted food, so that IR control is not valid.

which are sudanophilic (fig. 13), Schiff-positive and autofluorescent are limited to the outermost portion of the fasciculata. Furthermore, the quantity of birefringent material is subnormal in rat C but still about normal in D and E, although in the latter 2 there are virtually no fine particles (fig. 14).

The livers of the deficient rats show no fatty infiltration, and no signs of liver abnormality appear, except that the cells are smaller than normal.

c. Weight restricted and intake restricted rats. No essential differences occur between those rats that were paired with the deficient rats for weight change (WR) and those that were paired for food intake (IR). In addition to an adrenal hypertrophy like that of the deficient rats (but less atrophy of the thymus), the fasting rats possess adrenals that evince a similar but less extreme alteration in ketosteroid content which can be briefly summarized as follows. Throughout the experimental period the lipid of the glomerulosa remains unchanged in quantity and histochemical reaction. In the fasciculata, however, the sudanophilic lipids first rise above normal and then decline somewhat, especially in the small animals (figs. 9 and 11). The ketosteroids appear normal at the end of 1 week, increased in amount at 2 weeks, and greatly augmented the third week in D and E, though slightly reduced in C. The superabundant birefringent material in the fasciculatas of the larger animals at 3 weeks is illustrated in figures 10 and 12.

The livers of these 10 rats present no signs of any abnormality.

DISCUSSION

Thiamine deficiency, inanition, and the adaptation syndrome

Dietary deficiencies of 4 B-vitamins have now been studied with respect to their effect on the adrenal cortex and thymus of the rat. Two of these deficiencies, pantothenic acid and thiamine, cause a stimulation and ultimate exhaustion of the zona fasciculata of the adrenal cortex and a concomitant atrophy of the thymus. Stimulation of the adrenal cortex in

advanced thiamine deficiency has also been reported for the dog (Goodsell, '41a, b). In thiamine deficient rats the final exhaustion of the adrenal cortex may be gauged not only by the disappearance of the ketosteroid reactions from the zona fasciculata, but by such features as the abnormally swollen mitochondria in the fasciculata cells and the subnormal quantity of glycogen in the livers. We have found that the effect of thiamine deficiency is more rapid and more severe than that of a comparable degree of inanition, during which the adrenals are stimulated but do not become exhausted so quickly.

The reactions of the adrenals and thymus to these 2 B-vitamin deficiencies and to inanition fulfill the definition of the adaptation syndrome (Selye, '46). In this syndrome the production and release of the cortical steroids (probably those with an oxygen on C_{11}) is stimulated by adrenotropin during physiological stress. These cortical hormones in turn increase protein catabolism and cause thymus involution (Dougherty and White, '45).

One consideration which emerges from the present study is the importance of the relative and progressive nature of the adrenal changes. These changes occur more rapidly with severe stress than they do with mild insults. Furthermore, stress of a given intensity seems to affect young animals more severely than it does older ones. Moderate stress leads to continued resistance, with an elevated content of adrenal steroids, and severe stress causes subsequent exhaustion of the adrenal. These stages of adrenal reactivity must be taken into account in evaluating experimental studies on the activity of the adrenal cortex. For example, in 1 such study, Foster et al. ('44) tested the resistance of thiamine deficient mice and pair-fed controls toward poliomyelitis. Between 10 and 20 days on the diets the deficient mice were more resistant to the disease than the inanition controls, and both were more resistant than *ad libitum* controls. Similarly Murray and Morgan ('46) observed an enhanced resistance to anoxia in guinea pigs fed a vitamin C deficient diet for 15 to 30 days in comparison to pair-fed controls. On the other hand, Smith, Oster

and Toman ('44) found thiamine deficient cats less resistant to low barometric pressure than starved controls. All such differences need to be related to the degree of adrenal stimulation. One study which has attempted to trace the course of adrenal changes in stress is that of Oleson and Bloor ('41). These investigators analyzed the adrenals of rabbits starved from 3 to more than 14 days. At 3 days the adrenal weights were below normal, although the steroid and triglyceride contents were elevated. Later the weight of the glands rose steadily and the percentage of steroid remained above normal while the triglyceride content fell. These chemical analyses provide further interest since they correlate with our observation that sudanophilia and the steroid reactions may change independently.

Riboflavin and pyridoxine deficiencies. In contrast to deficiencies of pantothenic acid or thiamine, the dietary absence of riboflavin or pyridoxine fails to produce the adaptation syndrome. Physiological tests have similarly failed to show adrenal stimulation in riboflavin deficiency (Wickson and Morgan, '46). Early in these 2 deficiencies a mild stimulation of the adrenal cortex occurs, but this later disappears. Possibly this transitory stimulation results from the moderate inanition induced in these animals, to which they later become adjusted. The time of stimulation coincides with that of the most acute signs of deficiency (p. 4).

In pyridoxine deficiency, however, even without signs of persisting adrenal activity, severe thymus atrophy occurs, as has also been reported by Stoerk and coworkers ('44, '46). Consequently thymus involution need not always be attributed to a high level of secretion of the corticosterone-like hormones. Perhaps this atrophy results from inadequate protein synthesis.

If the 4 deficiencies that have been studied were of similar severity, the hypothesis would seem to be invalid that the stimulus for the release of extra adrenotropin might be an upset in carbohydrate oxidation, since a riboflavin deficiency does not produce continuing adrenal stimulation. Therefore,

at present the most attractive hypothesis to explain the release of adrenotropin is that the lack of thiamine or pantothenic acid causes organ changes which increase the utilization of C_{11}-oxygenated hormones (see, for example, Ralli, '46). If so, what such changes may be remains to be elucidated.

SUMMARY AND CONCLUSIONS

Severe dietary deficiencies of thiamine, riboflavin and pyridoxine were produced in weanling male rats. Weight changes of the adrenals and thymus glands, and cytochemical studies of the ketosteroids in the adrenal cortices show that the lack of thiamine causes stimulation of the zona fasciculata of the adrenal cortex in 2 weeks and its exhaustion in about 4 weeks in a fashion comparable to that induced by pantothenic acid deficiency (Deane and McKibbin, '46). These changes are attributed to a stimulation of the adrenal cortex by adrenotropin, resulting in an increased production and secretion of the corticosterone-like hormones. The latter hormones in turn produce thymus involution. In a paired feeding experiment it was found that stimulation of the fasciculata occurs earlier in thiamine deficiency than with a comparable degree of inanition.

On the other hand, neither a riboflavin nor a pyridoxine deficiency causes more than a transitory stimulation of the adrenal cortex, although a lack of pyridoxine results in acute involution of the thymus.

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Photomicrographs of adrenal cortices from glands fixed in 10% neutralized formalin for 2 days or more, then sectioned on the freezing microtome at 15 μ. Sections mounted in glycerin jelly.

PLATE 1

EXPLANATION OF FIGURES

All figures × 135. Demarcation between cortex and medulla of adrenal drawn in.

1–3 Stained with sudan IV and Harris' hematoxylin. Photographed with blue filter (Wratten H) to accentuate red stain.

1 First experiment; control rat, D, fed the control purified diet for 6 weeks. This figure shows the usual distribution of sudanophilic lipids in the adrenal cortex: rich in the cells of the zona glomerulosa, absent from the transitional zone, rich again in the outer part of the zona fasciculata, dwindling in the inner fasciculata, and variably present in the cells of the juxtamedullary zona reticularis.

2 Second experiment; thiamine deficient rat, A, fed the deficient diet for 1 week. The glomerulosa appears essentially normal; the outer fasciculata cells have watery vacuoles, and the whole fasciculata appears somewhat depleted of lipids.

3 First experiment; thiamine deficient rat, E, fed the deficient diet for 4½ weeks. The animal was moribund when sacrificed. The entire fasciculata is exhausted of lipids, although the glomerulosa contains its usual complement. The cells of the outer fasciculata appear "vacuolated." The reticular arrangement of the cell cords appears to be extended outward.

4–6 Stained by Schiff plasmal method. Photographed with combined green and yellow filters (Wratten B and G) to accentuate the fuchsin stain.

4 Second experiment; *ad libitum* rat, C, fed the control, purified diet for 3 weeks. The Schiff-positive material is normally distributed in the same fashion as is that of the sudanophilic lipids.

5 First experiment; riboflavin deficient rat, B, fed the deficient diet for 6 weeks. The usual quantity of Schiff-positive material is present in the cortex, although the transitional zone has been obliterated.

6 First experiment; pyridoxine deficient rat, C, fed the deficient diet for 8 weeks. The distribution of Schiff-positive droplets is entirely normal.

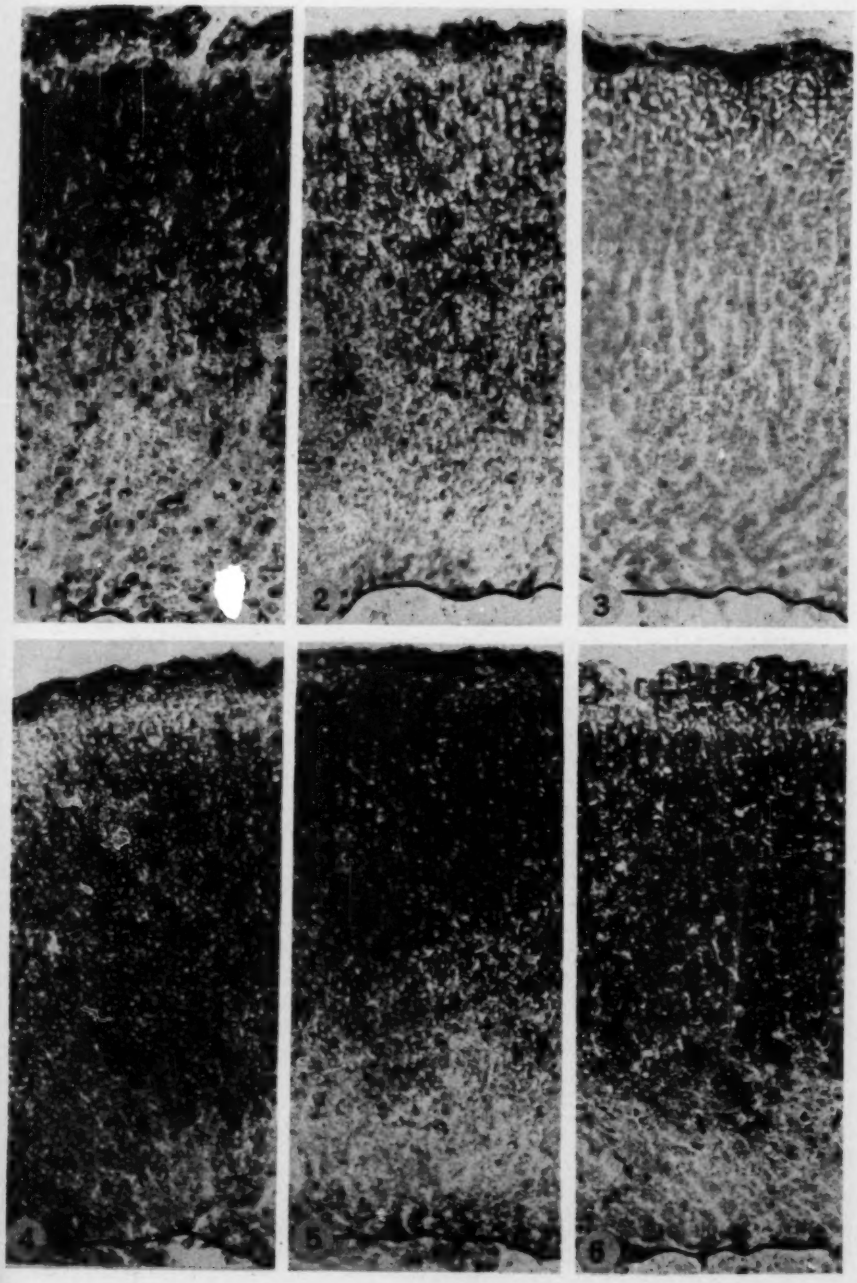
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PLATE 2

EXPLANATION OF FIGURES

Second experiment, comparing the effect of thiamine deficiency with that of inanition on the steroids in the adrenal cortex. Figures 7 and 8, rats fed the control purified diet *ad libitum*; figures 9 and 10, intake restricted rats; figures 11 and 12, weight restricted rats; figures 13 and 14, thiamine deficient rats. Of each pair, the figure on the left is from the smaller animal, C, and is stained with sudan IV and Harris' hematoxylin, × 113. The figure on the right is from a larger, less severely affected rat, and demonstrates the birefringent lipids which are present in unstained sections. × 100.

7 *Ad libitum* control, C. Normal distribution of sudanophilic lipids. As is often true, lipids in the fasciculata stain more lightly with sudan IV than do those in the glomerulosa.

8 *Ad libitum* control, D. Predominantly fine birefringent particles occur in the glomerulosa, and mixed coarse and fine particles are present in the fasciculata.

9 Intake restricted rat, C. The transitional zone has been obliterated and there has been some depletion of the lipids from the fasciculata.

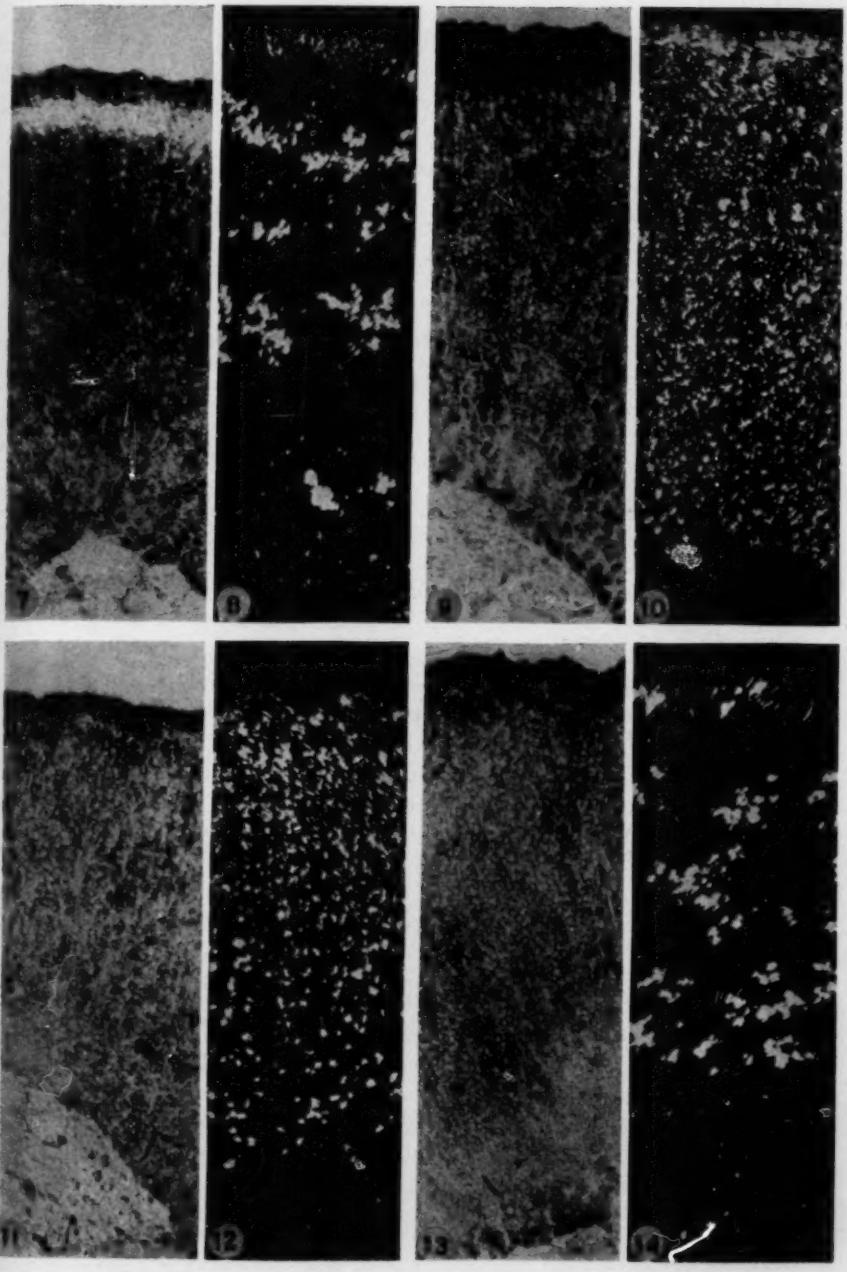
10 Intake restricted rat, D. Great augmentation in the amount of birefringent lipids in the fasciculata. This section exemplifies the "resistance stage" of Selye's adaptation syndrome.

11 Weight restricted rat, C. Marked depletion of lipids from fasciculata — near exhaustion.

12 Weight restricted rat, E. Supranormal content of birefringent material in the zona fasciculata, although the content is less than that illustrated in figure 10.

13 Thiamine deficient rat, C. Virtual exhaustion of the fasciculata.

14 Thiamine deficient rat, D. Subnormal amount of birefringent material in the fasciculata, particularly of the fine particles, which appear only in the inner fasciculata.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FURTHER STUDIES OF MANGANESE DEFICIENCY IN THE RABBIT

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In a previous paper from this laboratory (Smith, Medlicott and Ellis, '44), a number of differences between control and manganese deficient rabbits were described. The diets were fed ad libitum and some of the differences noted might well be ascribed to a difference in food intake, particularly since the growth rate of the control group was appreciably greater than that of the experimental group. By equalizing feed intake, those differences due specifically to a lack of manganese can be more readily differentiated from those due to a lack of other factors. The paired-feeding technique (Mitchell and Beadles, '30) was therefore used in the present study. A miscellany of results of several other manganese deficiency experiments which have not been reported elsewhere are also presented.

EXPERIMENTAL

Dutch rabbits were bred to furnish young for the experiment. To prevent access to feed high in manganese, the stock diet was replaced by milk and milk powder when the young were 2 weeks of age, and the dams moved to another cage for 2 hours each day in which they were fed a stock diet of mixed grains and hay. The young were weaned at 3 weeks of age and placed on the experiment proper. Twelve pairs were selected, each consisting of litter mates of the same sex and

of nearly the same body weight. The rabbits were kept in individual wire-screened cages and fed a basal low-manganese diet of milk plus 10% of whole milk powder. Sufficient iron and copper were added to the diet to give a mean daily intake of 4 mg of iron and 0.4 mg of copper per animal. These supplements were fed twice weekly as the chlorides, prepared from redistilled HCl and carbonyl iron and electrolytic copper. One member of each pair was also given 2 mg of manganese (a solution of c.p. $MnCl_2 \cdot 4H_2O$) per day by mouth. The animals were weighed weekly until 17 weeks of age, at which time they were autopsied. Various tissues were taken as indicated below.

Three rabbits died before completion of the experiment. Two of these were a pair, while the third was on the low-manganese diet. Thus 10 pairs of rabbits were available for observation at the end of the experiment. The calcium and phosphorus content of the bone ash was determined by the methods of Morris, Nelson and Palmer ('31). The methods used to determine hemoglobin, liver manganese, and the ash, density, and breaking strength of the bones have been described (Smith, Medlicott and Ellis, '44). The inorganic phosphorus content of blood serum was determined by the method of Fiske and Subbarow ('25) using a photoelectric colorimeter. The "alkaline" phosphatases were determined by the King and Armstrong ('34) method at a pH of 9.8 (sodium barbital buffer), while the "acid" phosphatases were determined by the Gutman and Gutman ('40) procedure at a pH of 5.3 for all tissues except blood serum, which was run at a pH of 4.9. Tyrosine was used as the primary standard as recommended by Greenberg et al. ('40), but the results are expressed in terms of phenol, each unit being the amount of enzyme that liberates 1 mg of phenol in 1 hour under the prescribed conditions. The arginase values were obtained by a modified combination of the Edlbacher and Röthler ('25). and Lightbody ('38) methods as described by Kochakian ('44). Incubation was for a period of 4 hours at 37°C. and at a pH of 9.2. A unit is defined as that amount of enzyme pro-

ducing 1 millimol of urea in 4 hours under the prescribed conditions.

Redistilled water was used in the preparation of the substrates for both the arginase and phosphatase determinations as well as in the preparation of the tissue extracts. The soft tissues were homogenized according to the method of Potter and Elvehjem ('36), while the bones were ground with sand and water in a glass mortar and pestle. Where the bone ends and shafts were run separately, the division was made according to length, the 2 end quarters being combined. Three per cent of toluene by volume was added to the unfiltered extract, which was stored in the coldroom for 12–16 hours before an aliquot was removed for the determination.

The manganese determinations on blood serum and on the bones were made by the method of Gates and Ellis ('47).

The testes were fixed in Susa's fluid, imbedded in paraffin, sectioned at 8 μ, and stained with iron hematoxylin and eosin.

RESULTS OF THE PAIRED-FEEDING TRIAL

The rabbits fed the manganese-low milk diet showed the same gross symptoms previously described (Smith, Medlicott and Ellis, '44) whereas the rabbits fed the additional supplement of manganese were normal. Six of the 10 rabbits fed the milk diet showed gross crookedness of the front legs, which was confirmed by observation of the dissected bones. As in previous experiments, the bending was confined to the radius and ulna.

A comparison of the growth of control rabbits with those on the manganese-deficient diet is given in table 1. Though the food intake of the 2 groups was held constant, the control rabbits gained weight at a significantly higher rate than those fed the deficient diet, showing that a deficiency of manganese per se retards growth.

A summary of the studies on the bones is presented in table 2. The fresh weight, percentage of ash, total ash, density and length of the humeri of the manganese-deficient rabbits were significantly less than those of the controls. The volume

TABLE 1

Growth of pair-fed control and manganese-deficient rabbits.

TREATMENT	NO.	AGE AT AUTOPSY	MEAN DAILY FEED INTAKE	MEAN INITIAL WEIGHT	MEAN FINAL WEIGHT
		wks.	*ml*	*gm*	*gm*
Control	5 [1]	17	58.6	282	1240 [2]
Mn deficient	5	17	58.2	279	1053 [2]

[1] By error final weights obtained on only 5 of the 10 pairs.
[2] Difference significant at odds of 19: 1.

TABLE 2

Tissue composition of pair-fed control and manganese-deficient rabbits.

	CONTROL [1]	MN DEFICIENT [1]	MEAN DIFF. AND S.E.
Humerus			
Fresh wt. (gm)	1.954	1.809	0.145 ± .0391 [2]
Ash (%, dry fat-free bone)	58.88	57.03	1.85 ± .714 [2]
Ash (total)	0.5648	0.4702	0.0946 ± .02154 [2]
Volume (ml)	1.479	1.409	0.070 ± .034
Density (gm/ml)	1.318	1.282	0.036 ± .0148 [2]
Length (mm)	51.1	45.6	5.5 ± 1.22 [2]
Breaking strength (lbs)	32.3	33.3	−1.0 ± 1.20
Ca (%, dry fat-free bone)	22.47	21.95	0.52 ± .286
P (%, dry fat-free bone)	10.67	10.22	0.45 ± .109 [2]
Ulna			
Fresh wt. (gm)	0.766	0.725	0.041 ± .0274
"Alkaline" Phosphatase (units/gm fresh wt.)			
No activation	25.9	12.7	13.2 ± 1.36 [2]
Activation with Mn	30.9	15.0	15.9 ± 1.19 [2]
Femur			
Fresh wt. (gm)	3.804	3.433	0.371 ± .0827 [2]
Dry fat-free wt. (gm)	1.934	1.592	0.342 ± .0558 [2]
Dry fat-free wt (% fresh bone)	50.3	45.8	4.5 ± 1.0 [2]
Mn (μg/gm dry fat-free bone)	6.98	0.52	6.46 ± 1.136 [2]
Liver			
Dry matter (%)	32.2	29.9	2.3 ± .91 [3]
Mn (μg/gm — dry basis)	6.04	0.43	5.61 ± .793 [2]
Arginase (units/gm fresh tissue)			
No activation	20.5	15.4	5.1 ± 1.95 [3]
Activation with Mn	25.6	19.2	6.4 ± 2.73 [3]
Blood Serum			
Mn (μg/100 ml)	4.8	2.0	2.8 ± 1.57

[1] Mean values of 10 rabbits.
[2] Difference significant at odds of 99: 1.
[3] Difference significant at odds of 19: 1.

of the humeri of the 2 groups did not differ significantly. These observations confirm those previously found in experiments where food intake was not controlled. The breaking strength of the humeri of the 2 groups was found not to be significantly different, which varies from previous observations in which the diets were fed ad libitum. Further studies have indicated that the breaking strength of the bones of rabbits in these experiments is closely correlated with body weight. In another experiment (Smith and Ellis, '47), rabbits were fed no manganese, 0.3, 0.6, 1, or 8 mg of manganese per day. Within each treatment group the correlation coefficients between the breaking strength of the humeri and body weight were respectively, 0.73, 0.78, 0.69, 0.24, and 0.83. It thus appears that the breaking strength of bone is more closely associated with growth than with a deficiency of manganese per se.

Phosphorus and calcium determinations were made on the ash obtained from the humeri. No significant difference was observed for calcium, but the percentage of phosphorus in the dry, fat-free bones of the manganese-deficient rabbits was slightly but significantly lower than the controls. The meaning of this is obscure.

In this experiment the only tissue for which phosphatase values were obtained was the ulna. In one case the substrate used in the determination was made 0.00004 M with respect to Mn^{++}, while in the other no Mn was added. In both cases, the "alkaline" phosphatase activity was approximately one-half as great in the deficient as in the control group.

The fresh and the dry fat-free weight of the femur were both greater in the control group, and this was also true of the percentage of dry fat-free bone. The manganese content of the femurs was approximately 13 times higher in the group given manganese than in the group not receiving it.

The dry matter, manganese content and arginase activity of the liver of the deficient group were all lower than in the controls. As in the phosphatase determination, the arginase activity was determined both with and without activation by

Mn. In this case the substrate was made 0.0004 M with respect to Mn^{++}.

In contrast to the marked reduction in the manganese content of the bone and liver of animals on the low-manganese diet, the reduction in serum manganese from 4.8 to 2 mg/100 ml was not significant at odds of 20 to 1. This is in agreement with the findings of Johnson ('40) that the level of manganese intake does not affect the blood values in either sheep or pigs.

RESULTS OF OTHER EXPERIMENTS

Phosphatase determinations were made on various tissues obtained from rabbits on 2 experiments in both of which the milk diet with and without a supplement of manganese was fed ad libitum. In one experiment the manganese was fed at 0, 1, 2, and 4 mg per day levels (Smith and Ellis, '47), and in the other 0 and 4 mg per day. There was no significant difference in either "acid" or "alkaline" phosphatase activity of the blood serum, kidney, liver, small intestine, and none in the "acid" phosphatase of the ulna. There was a significant difference in the "acid" phosphatase of the serum in one experiment, but this was not confirmed by the second experiment. The results are summarized in table 3. The number of animals is rather small to demonstrate conclusively that there are no differences, but it is clear that differences, if they exist, are much smaller than for the "alkaline" phosphatase activity of the bones.

Inorganic phosphorus determinations were made on the blood serum of the 0, 1, 2, and 4 mg per day rabbits with negative results as shown in table 3.

In previous work (Smith et al., '44) no change in the hemoglobin level of manganese-deficient rabbits was found, and this observation has been confirmed with larger numbers. In 62 rabbits (Smith and Ellis, '47) fed either the manganese-deficient diet alone or supplemented with 1, 2, or 4 mg of manganese per rabbit per day, the mean range of hemoglobin

TABLE 3

Phosphatase values for various tissues of manganese-deficient and of control animals.

	LEVEL MN FED MG/DAY					
	0	1	2	4	0	4
No. of Animals	*10*	*9*	*9*	*8*	*8*	*7*
Inorganic P Serum (mg/100 ml)	7.36 ± .56 [1]	9.26 ± .84	8.41 ± .42	6.65 ± .99	.....	.....
Phosphatase						
Blood Serum (units/100 ml)						
"Acid"	10.2 ± 1.24	14.9 ± .88	14.7 ± .99	14.4 ± .71	11.7 ± 1.43	12.1 ± .81
"Alkaline"	24.4 ± 1.80	25.2 ± 3.44	25.7 ± 2.90	24.3 ± 1.92	25.8 ± 2.38	25.1 ± 1.12
No. of Animals	*6*	*4*	*9*	*3*	*4*	*4*
Kidney [2]						
"Acid"	8.2 ± 1.00	8.6 ± .31	9.4 ± .55	9.6 ± .61	.....	.....
"Alkaline"	49 ± 5.0	61 ± 14.6	65 ± 7.2	83 ± 23.4	48 ± 10.4	65 ± 10.1
Liver [2]						
"Acid"	6.2 ± 1.48	6.1 ± .16	6.7 ± .41	6.0 ± .60	.....	.....
"Alkaline"	27 ± 5.6	19 ± 3.5	17 ± 1.7	19 ± .8	14 ± 3.9	16 ± 3.4
Small Intestine [2]						
"Acid"	5.4 ± .89	8.2 ± .74	8.6 ± .34	8.1 ± .98	.....	.....
"Alkaline"	60 ± 18	101 ± 22	95 ± 11	104 ± 14	71 ± 19.0	42 ± 14.1
Bone End [2] (ulna)						
"Acid"	2.6 ± .61	2.9 ± .75	1.7 ± .47	1.8 ± .42	.....	.....
"Alkaline"	9.3 ± 1.49	32.5 ± 2.62	27.8 ± 3.49	22.9 ± 2.95	19.6 ± 3.7	32.7 ± 6.9

[1] Standard error.

[2] Units/gm fresh tissue.

values per treatment group was 10.47–11.22 gm per 100 ml of blood. These differences are not significant.

The effect of a deficiency of manganese on the testes of rabbits has been more thoroughly studied. Two out of 9 male rabbits fed the manganese-deficient diet showed a mild tubular degeneration after being fed the deficient diet for 12 to 22 weeks. Two of 12 comparable males fed manganese supplements (1, 2, or 4 mg per day) in addition to the basal diet also showed mild tubular degeneration. These observations indicate that a deficiency of manganese does not lead to testicular degeneration in the rabbit as it apparently does in the rat (Shils and McCollum, '43; Boyer, Shaw and Phillips, '42).

DISCUSSION

The rat (Shils and McCollum, '43; Boyer, Shaw and Phillips, '42) and the rabbit show a similar decrease in liver arginase activity both with and without manganese activation on a low-manganese diet when arginine monohydrochloride is used as the substrate in the arginase determination. Shils and McCollum found that when arginine carbonate was used as the substrate no difference in enzyme activity between manganese-deficient and control rats was found when manganese was used to activate the system, although there was a marked difference when no manganese was added. In view of this finding it is not possible to conclude that in manganese deficiency in the rabbit there is a reduction of liver arginase concentration.

The activation of "alkaline" phosphatase of the chick bone noted by Wiese et al. ('39) was confirmed both for the "acid" and "alkaline" phosphatase of the serum and for the "alkaline" phosphatase of the bone in the rabbit. These determinations were made both with and without manganese activation as an aid in the interpretation of the results. Since the differences noted occurred in both instances, the evidence suggests that the loss of enzyme activity is not due to a lowered concentration of manganese in the bone per se. This evidence, however, cannot be taken as conclusive since

manganese added in the determination may not activate the enzyme to the same extent as manganese found in vivo.

In making an interspecies comparison of the effects of manganese deficiency, it is interesting to note that in the chick there is a reduced "alkaline" phosphatase activity of the tibia and of the blood serum (Wiese et al., '39; Combs, Norris and Heuser, '42), while in the rat Amdur, Norris and Heuser ('45) found a small, but highly significant reduction of the "alkaline" phosphatase of the bones in manganese deficiency contrary to the findings of Wachtel, Elvehjem and Hart ('43). In the rabbit, the "alkaline" phosphatase of the bone is reduced, as in the chick and rat.

There is growing evidence also that the skeletal development is impaired in the rat and mouse (Shils and McCollum, '43; Amdur, Norris and Heuser, '45; Barnes, Sperling and Maynard, '41; Wachtel, Elvehjem and Hart, '43), in the chick (Caskey, Gallup and Norris, '39), and the rabbit (Smith, Medlicott and Ellis, '44), and is suggested by the results of Miller, Keith, McCarty and Thorp ('40) with swine. Thus in the rat, chick, and rabbit bone changes have been shown to be associated with a decreased "alkaline" phosphatase activity of the bones in manganese deficiency, and it seems clear that manganese is concerned specifically in bone formation probably through its influence on phosphatase activity.

Likewise, growth has been shown to be retarded in the manganese-deficient chick, rat, mouse, and rabbit by these workers.

Other symptoms of manganese deficiency, such as testicular degeneration, impaired lactation, and nervous disorders have been found less consistently and may reflect a varying severity of the deficiency.

SUMMARY

Manganese deficiency in the rabbit was studied using the paired-feeding technique with a basal diet of milk.

On the unsupplemented diet there was a lowered arginase activity of the liver and a lowered "alkaline" phosphatase activity of the ulna. The latter finding is associated with

impaired bone formation noted in the gross as crooked front legs. The fresh weight, percentage ash, total ash, density and length of the humerus was significantly lower in the manganese-deficient animals.

Though the food intake of the 2 groups was equalized, the controls gained more weight than those fed the low-manganese diet.

In the manganese-deficient animals the concentration of manganese in the liver and in the femur was much reduced, while that in the blood serum was not.

In other experiments in which the feed intake was not equalized, no significant differences were found for the following: "acid" or "alkaline" phosphatase activity of the blood serum, kidney, liver, or small intestine; hemoglobin concentration of the blood; inorganic phosphorus of the serum; incidence of testicular degeneration.

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STUDIES OF THE MANGANESE REQUIREMENT OF RABBITS

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The manganese requirement has been established for poultry (Wilgus, Norris and Heuser, '39), but for no other animal insofar as the authors are aware. Following the background studies which characterized the symptoms of manganese deficiency in the rabbit (Smith, Medlicott and Ellis, '44), it became possible to determine the minimum amount of manganese required to prevent these symptoms. This paper summarizes the results of such studies.

Knowledge of the requirement of any nutrient for various metabolic processes is essential as a baseline for assessing the adequacy of a feed or ration for that nutrient. It is realized that any suggested value is subject to variation dependent on many factors. Among such factors are the availability of the nutrient which is a function of the chemical form in which it is fed as well as of the effects of other components of the diet. The requirement is also subject to genetic variability as is often noted in breed or strain differences, the age of the animals, and the criteria used to determine the adequacy of the nutrient levels fed. The established deficiency of a milk diet with respect to manganese, the general adequacy of a milk diet in nutrients other than manganese, iron, and copper, and a lack of knowledge of a

practical ration of forages and concentrates sufficiently low in manganese to serve as a basal diet prompted the use of milk and milk products in this study.

MATERIALS AND METHODS

The technics and materials used in this study have been outlined in detail in a previous publication (Smith et al., '44). The basal diet was fed ad libitum and consisted of whole milk powder [1] supplemented with highly purified iron and copper. Each rabbit received 2 mg of iron and 0.2 mg of copper daily in a little fluid milk. Manganese ($MnCl_2$, c.p.) was fed by mouth daily to those rabbits indicated in the tables. Distilled water was provided in pyrex glass cups. All rabbits were weighed at weekly intervals and carefully observed for symptoms.

The manganese content of the whole milk powder was determined by the method of Gates and Ellis ('47). Samples were taken in duplicate from each of 5 cans. The average value for the duplicates ranged from 0.109 to 0.191 μg per gm. The mean value for the 5 cans was 0.141 μg per gm.

Two experiments were conducted at different times. The first experiment was exploratory, while the second experiment was designed to determine more precisely the quantity of manganese required to prevent the various symptoms. In the first experiment 39 rabbits of mixed sexes and ranging in age from 21 to 24 days of age were divided into 4 groups of 12, 9, 9, and 9 rabbits each. In addition to the above basal diet the groups were fed, respectively, 0, 1, 2, and 4 mg of manganese per rabbit per day. Among those rabbits fed the basal diet alone, 3 died at the respective ages of 10, 12, and 15 weeks. One rabbit in the 4 mg manganese-supplemented group died at 10 weeks of age. The remaining rabbits, with the exception of 4 females fed a 1-mg supplement of manganese and 4 females fed a 4-mg supplement of manganese, were sacrificed at 19 weeks of age. The 8 female rabbits were

[1] Borden's Klim.

continued on the experiment to observe the effects of manganese deficiency on reproduction.

In the second experiment 45 rabbits were distributed at random among 5 equal groups and fed the basal diet supplemented, respectively, with 0, 0.3, 0.6, 1, and 8 mg of manganese per rabbit per day. These rabbits were sacrificed at 20 weeks of age with the exception of 11 that died earlier in the experiment. These 11 rabbits were distributed at random among the groups receiving treatment. These deaths occurred for the most part in the late stages of the experiment (see discussion).

The deficiency symptoms observed in previous studies (Smith et al., '44; Ellis, Smith and Gates, '47) were used as criteria to determine the adequacy of the various levels of manganese fed, namely, gross crookedness of the front leg bones, subnormal growth, length, density and weight of the bones, manganese concentration of the bones and livers, "alkaline" phosphatase activity of the bones and arginase activity of the livers.

The chemical methods used to determine manganese, "alkaline" phosphatase activity and arginase activity are described in detail by Ellis et al. ('47).

The data obtained were reduced by means of the analysis of variance and the *t* test used to determine whether particular differences were significant. Odds greater than 19:1 were accepted as statistically significant while odds greater than 99:1 were considered highly significant. The data presented in this paper are in all cases mean values with their standard errors.

RESULTS

In both experiments most of the rabbits fed the basal manganese-low diet developed crooked front legs. In the first experiment 7 out of 12 and in the second experiment 7 out of 9 developed crooked front legs that were evident in the intact animals and confirmed by the observation of a bending of the radii and ulnae in the dissected bones. No animal

which received 0.3 mg of manganese per day or more showed any sign of crooked leg bones. Microscopic examination of the humeri bones from those rabbits fed the basal diet showed evidence of depressed bone formation previously described whereas the bones from rabbits fed 0.3 mg of manganese or more were to all appearances normal.

Table 1 summarizes the observations on the length, density and fresh weight of the humeri bones of the various treat-

TABLE 1

Summary of the observations on the bones of rabbits fed various levels of manganese.

MN FED	ANIMALS	AV. LENGTH OF HUMERI	AV. DENSITY OF HUMERI	AV. FRESH WT. OF HUMERI
mg/day		*cm*	*gm/ml*	*gm*
		First experiment		
0	11	4.87 ± 0.101	1.329 ± 0.0194	1.988 ± 0.1479
1	5	5.36 ± 0.163	1.366 ± 0.0133	2.191 ± 0.1878
2	9	5.53 ± 0.101	1.399 ± 0.0119	2.416 ± 0.0881
4	4	5.21 ± 0.121	1.361 ± 0.0507	2.093 ± 0.2839
		Second experiment		
0	7	4.81 ± 0.123	1.331 ± 0.0277	2.199 ± 0.1370
0.3	7	5.27 ± 0.097	1.385 ± 0.0146	2.437 ± 0.1013
0.6	7	5.31 ± 0.152	1.374 ± 0.0164	2.232 ± 0.0834
1	7	5.56 ± 0.110	1.375 ± 0.0094	2.579 ± 0.0775
8	6	5.55 ± 0.026	1.370 ± 0.0159	2.367 ± 0.0507

Analysis of variance showed a highly significant or significant (fresh weight of humeri) difference among the treatment groups in each experiment.

ment groups. In both experiments the length, density and fresh weight of the humeri of the rabbits fed the basal diet were significantly less than the respective values for rabbits fed additional manganese. No significant improvement in bone length, density or weight was obtained by feeding more than 0.3 mg of manganese per day.

The manganese concentration and the "alkaline" phosphatase activity of the ulna bones are given in table 2. The manganese content per unit of dry, fat-free bone was very low in those rabbits fed the basal diet alone as compared with

those rabbits fed additional manganese. The manganese concentration continued to increase with increasing manganese intake within each experiment. It will be noted that the manganese concentration of the bones of the rabbits fed 8 mg of manganese in the second experiment is less than in those rabbits fed 4 mg of manganese in the first experiment. Since these experiments were conducted at different times and with

TABLE 2

The manganese concentration and alkaline phosphatase activity of the bones of rabbits fed various levels of manganese.

MN FED	NO. OF ANIMALS	AV. MN CONTENT [1] OF FEMUR	NO. OF ANIMALS	AV. "ALKALINE" PHOSPHATASE ACTIVITY OF ULNAE
mg/day		*μg/gm*		*Units/gm* [2]
		First experiment		(ends of bones) [3]
0	4	0.60 ± 0.080	6	9.3 ± 1.49
1	4	3.07 ± 0.319	4	32.5 ± 2.62
2	9	5.68 ± 0.668	9	27.8 ± 3.49
4	3	12.91 ± 0.322	3	22.9 ± 2.95
		Second experiment		(whole bones)
0	7	0.44 ± 0.052	6	5.4 ± 0.70
0.3	7	2.26 ± 0.185	7	10.4 ± 1.59
0.6	7	2.48 ± 0.270	7	9.3 ± 0.70
1	7	2.76 ± 0.170	7	9.2 ± 1.18
8	5	6.71 ± 1.350	6	10.5 ± 1.12

Analysis of variance showed a highly significant or significant (phosphatase activity in second experiment) difference among the treatment groups in each experiment.

[1] Dry, fat-free bone. [2] Fresh bone. [3] The 2 end quarters.

different groups of rabbits, one may question whether this is a real difference. The "alkaline" phosphatase activity per unit weight of fresh bone was significantly less in the rabbits fed the basal diet alone as compared to those animals fed additional manganese. No further significant increase in phosphatase activity was observed by feeding more than 0.3 mg of manganese.

Liver samples from the rabbits of the second experiment were taken for arginase activity determinations. The livers from the rabbits fed the basal diet had a significantly lower activity than the groups fed additional manganese. The arginase activity levels of the 0, 0.3, 0.6, 1 and 8 mg groups were, respectively: 15.6 ± 1.42; 24.7 ± 3.05; 29.4 ± 4.05; 24.6 ± 3.97, and 31.1 ± 3.42 units per gm of fresh liver. At first observation the activity levels of the groups fed additional manganese would appear to be significantly different, but this was not true. The large standard errors indicate the large variability encountered.

TABLE 3

Manganese content of the livers of rabbits fed various levels of manganese.

MN FED	NO. OF ANIMALS	AV. MN CONTENT OF LIVERS
mg/day		*γ/gm of dry tissue*
0	10	0.9 ± 0.41
0.3	8	6.8 ± 1.37
0.6	7	9.8 ± 1.68
1	10	16.2 ± 3.05
2	9	20.5 ± 1.74
4	9	22.7 ± 2.61
8	4	15.4 ± 1.45

The concentration of manganese in the livers of the various treatment groups is summarized in table 3. The low concentration of manganese in the livers of the rabbits fed the basal diet alone is marked. The manganese concentration of the livers increased significantly with increased intakes of manganese up to a level of 1 mg of manganese per day after which the concentration leveled off. The concentration of manganese in the liver would thus appear to reach a maximum at much lower intakes of manganese than do the bones.

The data (table 4) relative to the manganese requirement for maximum growth are not adequate enough to warrant a conclusive statement. It will be noted that in the first ex-

periment where manganese levels of 0, 1, 2 and 4 mg are directly compared, maximum growth was obtained in rabbits fed 4 mg of manganese per day. In the second experiment where manganese levels of 0, 0.3, 0.6, 1 and 8 mg were directly compared, maximum growth occurred in those rabbits fed 1 mg of manganese. Since experiments 1 and 2 were conducted at different times and with different rabbits they cannot logically be directly combined. This is supported by the observation that the rabbits fed 1 mg of manganese in the

TABLE 4

Growth of rabbits fed various levels of manganese.

MN FED	NO. OF ANIMALS	AV. INITIAL WEIGHT	AV. FINAL WEIGHT
mg/day		*gm*	*gm*
		First experiment	
0	9	288	1140 ± 52
1	9	285	1130 ± 107
2	9	288	1240 ± 82
4	8	282	1390 ± 63
		Second experiment	
0	7	274	990 ± 90
0.3	7	269	1290 ± 91
0.6	7	290	1194 ± 47
1	7	276	1350 ± 39
8	7	284	1120 ± 93

Analysis of variance showed a highly significant difference among the treatment groups in each experiment.

first experiment gained significantly less weight than those fed 1 mg of manganese in the second experiment. These limited observations indicate that the manganese requirement for maximum growth is higher than the requirement for normal bone development.

The fact that the rabbits fed 8 mg of manganese gained less weight than the rabbits fed 1 mg of manganese in the second experiment may indicate that 8 mg of manganese per day is on the borderline of toxicity for rabbits.

The manganese concentration of the blood serum was determined on pooled blood samples of the rabbits in the first experiment by the periodate method. In the second experiment the micromethod of Gates and Ellis ('47) permitted manganese to be determined on the blood serum of individual rabbits. Interpretation of the results is difficult because of the large variability observed and the details are not presented here but are being reserved for more study. In both experiments, however, the rabbits fed the basal diet alone, appeared to have significantly lower concentrations of manganese than the rabbits fed additional manganese. Whether or not a difference existed among rabbits fed varying levels of manganese is not certain.

DISCUSSION

The level of manganese taken as being adequate depends upon the criteria used. Insofar as crookedness, length, density, fresh weight, microscopic structure and "alkaline" phosphatase activity of the bones and arginase activity of the livers are concerned, 0.3 mg of manganese per rabbit per day appears to be adequate. The manganese concentration of the livers increased significantly up to an intake of 1 mg of manganese per day while the manganese concentration of the bones increased up to the highest level (8 mg) of manganese fed. The minimum manganese intake necessary for maximum growth requires more study, but it appears to be higher than the amount required for normal bone development.

The relatively high mortality of the rabbits, particularly in the second experiment, is worthy of further comment. This rate of mortality may have been influenced by a cirrhosis of the liver which we have consistently observed in rabbits fed a diet of milk or milk products. This cirrhosis was usually of a mild nature characterized by livers which were mottled and lighter in color than normal. In the extreme form the livers were a very light tan color, fibrous and fatty and apparently similar to the cirrhosis in rabbits described by Rich and Hamilton ('40). The incidence of cirrhosis was not cor-

related with the level of manganese intake. Studies designed to determine the cause of the cirrhosis are in progress.

SUMMARY

The manganese requirement of rabbits has been studied by feeding a basal diet low in manganese supplemented with various levels of manganese. Insofar as bone development is concerned, it appears that 0.3 mg of manganese per rabbit per day is sufficient to meet normal requirements. The minimum manganese intake necessary for maximum growth requires more study, but it appears to be higher than the amount required for normal bone development.

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SUPPLEMENTAL RELATIONSHIP BETWEEN PORK PROTEIN AND EGG PROTEIN

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The supplemental relationship of the protein in one food product to that in another is of great practical importance in human nutrition. In 1945 we reported that pork protein had a marked supplemental relationship to bread protein, and the question then arose as to the possibility of a similar relationship between pork protein and egg protein. Published data indicate a higher nutritive value for the protein in eggs than for that in pork. Mitchell, Beadles and Kruger ('27) reported a biological value of 79 for pork tenderloin, and Mitchell ('27) found a value of 74 for pork ham. Mitchell and Carman ('26) found a biological value of 94 for dried whole eggs, and 83 for egg albumin. Sumner ('38) found values of 97 for commercially-dried eggs fed to young rats and 85 for such eggs fed to year-old rats when the diet contained 8% of protein. When the diet contained 5% of protein, the biological value of these eggs fed to mature rats was 94. Sumner and Murlin ('38) reported a value of 95 for the protein in dried eggs fed to year-old rats when the diet contained 5% of protein, and a value of 65 when the eggs were consumed by human subjects on diets containing 3 to 4% of protein. Hoagland and Snider ('46) found the average growth-promoting value of the protein in dehydrated pork to be 3.35 gm gain per gm of protein consumed in tests with young rats, as com-

pared with a gain of 4.28 gm for the protein in spray-dried eggs.

The reason for the higher nutritive value of the protein in whole eggs than in pork is, presumably, a better balance between the essential amino acids in eggs and the requirements of the rat. A comparison of available data for the amino acid content of eggs with those for pork, as reported by Beach, Munks and Robinson ('43), Mitchell and Block ('46), and Lyman et al. ('46), indicates that the protein in whole eggs and in egg albumin contains notably more cystine and methionine than the protein in pork. It seemed probable, therefore, that there would be a supplemental relationship between the protein in eggs and that in pork. The purposes of the experiments herein reported were (1) to determine the growth-promoting values of the protein in mixtures of pork and eggs as compared with the values of each product alone, and (2) to determine the effects of the addition of cystine or methionine on the growth-promoting value of the pork protein.

EXPERIMENTAL

Products tested [1]

Three lots of cured and 1 lot of fresh hams were used in feeding tests with rats. Cured ham lot no. 4320 had been cured by the dry-cure method, and cured ham lots nos. 4462 and 4464 were mixtures of hams that had been cured by the dry-cure, artery-cure, and brine-cure methods. The lean meat from each lot of hams was ground and then baked in glass baking dishes in an oven at 325° to 350°F. until the internal temperature of the meat was 155°F. The cooked ham was ground, spread on trays, and dehydrated at 155°F. to a moisture content of 10% or less. The dried meat was thoroughly extracted with ethyl ether, ground fine, and stored in tightly covered jars at approximately 25°F.

[1] Acknowledgment is made to R. L. Hiner for curing and dehydrating the hams and for dehydrating the hard-boiled eggs and to Edna H. Steely for assistance in the statistical analyses. Our thanks are extended to T. C. Byerly and E. H. McNally for supplying fresh eggs and commercially dehydrated eggs.

Two lots of commercial spray-dried eggs (uncooked), 2 lots of dried hard-boiled eggs, and 1 lot of dried whites from hard-boiled eggs were tested. The dried hard-boiled eggs were prepared as follows: The eggs were boiled 10 minutes, chilled, and the contents were ground through a meat grinder, spread on trays, and dried in the same manner as the hams. The dried, cooked whole eggs were thoroughly extracted with ethyl ether, but the spray-dried eggs were not extracted. The dried eggs and egg white were ground fine and stored under the same conditions as the dried ham.

Diets fed

Dehydrated ham, eggs, and egg white were incorporated in diets in such proportions that each product or mixture supplied 1.6% of nitrogen. The following quantities of B vitamins were added to 100 gm of diet in addition to any vitamins present in the ham or eggs: thiamine hydrochloride, 0.3 mg; riboflavin, 0.3 mg; pyridoxine hydrochloride, 0.6 mg; calcium pantothenate, 1.5 mg; and choline chloride, 20 mg. To 1 diet containing spray-dried eggs was added 5 μg of biotin. Cystine and methionine were added to certain diets as indicated in the tables.

The fat-soluble vitamins were added in solution in lard so that 1 gm of the diet would contain 5 I.U. of vitamin A, 1 I.U. of vitamin D, and 20 μg of alpha-tocopherol. Sufficient kettle-rendered lard was added to make 10% of fat in the diet. Salt mixture amounting to 2%, sufficient sodium chloride to equalize the salt content of all diets in a series including cured ham, and dextrin to make 100% completed the diet. In experiment no. 4 (table 3) which did not include cured ham, 4% of salt mixture was added to the diet.

Feeding tests

Each diet was fed to 8 male albino rats for 30 days. The animals weighed approximately 40 gm each and did not exceed 25 days of age at the beginning of the tests. Rats from

different litters were distributed among the several groups in a series. Each rat was kept in an individual cage which was provided with a raised screen bottom, a self-feeder, and a drinking vessel. The bottom of the cage was covered with a sheet of blotting paper. The rats were weighed twice weekly and the feeders were refilled about every third or fourth day. A record was kept of the feed consumed. The temperature of the rat laboratory was approximately 75°F.

RESULTS

The results of experiment no. 1, table 1, indicate that the protein in 1 lot of spray-dried eggs was superior in growth-promoting value to the protein in dry-cured ham. This is true not only on the basis of gain in weight but more certainly on the basis of gain in weight per gram of nitrogen consumed, the criterion primarily employed in this present study for evaluating the diets. However, when the diet contained equal parts of ham and egg protein, the growth-promoting value was practically the same as for eggs alone, and definitely higher than the computed value. When the diet contained 2 parts of ham and 1 part of egg protein, the growth-promoting value was somewhat higher than the computed value, but lower than the value for eggs alone. The results of this experiment indicate a moderate supplemental relationship between the protein in dry-cure ham and that in cooked, spray-dried eggs.

Statistical tests by means of Fisher's "t" values for the significance of differences between the means of the gain in weight per gram of nitrogen consumed showed the following probability values:

Diet 1 with diets 2, 3, and 4	— very highly significant ($< .001$)
Diet 4 with diets 2 and 3	— highly significant ($< .01$)
Diet 2 with diet 3	— not significant ($> .6$)

In experiment no. 2, further tests were made on ham alone, eggs alone and combinations of ham and eggs, together with supplements of biotin, methionine and cystine added to cer-

TABLE 1

Supplemental relationship between the protein in cured ham and that in spray-dried eggs in 30-day tests with groups of 8 male rats.

SOURCE OF NITROGEN IN DIET	DIET NO.	GAIN IN WEIGHT		FEED CONSUMED	NITROGEN IN DIET	GAIN IN WEIGHT PER GRAM OF NITROGEN CONSUMED	
		Actual	Computed [1]			Actual	Computed [1]
		gm	*gm*	*gm*	%	*gm*	*gm*
Experiment no. 1 (dry-cured hams)							
Dry-cure ham no. 4320	1	105	...	320	1.60	20.48	....
Dried eggs no. 4343	2	111	...	285	1.60	24.26	....
Dry-cure ham no. 4320, 0.8% N + dried eggs no. 4343, 0.8% N	3	123	108	318	1.60	24.04	22.37
Dry-cure ham no. 4320, 1.067% N + dried eggs no. 4343, 0.533% N	4	117	107	324	1.60	22.62	21.74
Experiment no. 2 (hams cured by several methods)							
Cured ham no. 4462	1	101	...	316	1.60	19.93	....
Cured ham no. 4462 + 0.2% methionine	2	129	...	333	1.62	23.66	....
Dried eggs no. 4463	3	110	...	292	1.60	23.58	....
Dried eggs no. 4463 + 5 μg biotin in 100 gm diet	4	111	...	291	1.60	23.76	....
Dried eggs no. 4463 + 0.20% methionine	5	115	...	296	1.62	24.00	....
Cured ham no. 4462, 0.8% N + dried eggs no. 4463, 0.8% N	6	119	106	324	1.60	22.88	21.76
Cured ham no. 4462, 0.8% N + dried eggs no. 4463, 0.8% N + 0.2% cystine	7	130	106	326	1.62	24.51	21.76
Cured ham no. 4462, 1.067% N + dried eggs no. 4463, 0.533% N	8	116	104	333	1.60	21.53	21.06
Cured ham no. 4462, 1.067% N + dried eggs no. 4463, 0.533% N + 0.2% cystine	9	134	104	339	1.62	24.27	21.06

[1] The computed gain of rats fed a mixture of ham and eggs is the weighted average of gains made by rats fed the individual products.

tain ones of these diets. Statistical tests similar to those given for experiment 1 are summarized as follows:

Diet 1 with diets 2, 3, 4, 5, 6, 7, and 9 — very highly significant ($< .001$)
Diet 8 with diets 2, 3, 5, 7, and 9 — highly significant ($< .01$ or $.02$)
Diet 8 with diet 4 — significant ($< .05$)
Diet 6 with diet 7 — highly significant ($< .02$)
Diet 6 with diet 5 — significant ($< .05$)
Differences between all other diets — not significant.

The data given in table 1 indicate that the protein in a second lot of spray-dried (diet 3) eggs was also higher in growth-promoting value than the protein in a composite lot of cured hams (diet 1). However, when this lot of ham was supplemented with methionine, the nutritive value of the protein was increased to an approximate equality with the protein in eggs. It is noteworthy that the actual gain in weight of the rats on the diet containing ham and methionine was considerably larger than for the rats on the diet containing eggs alone. The addition of biotin to the egg diet was without material effect on growth, and the addition of methionine was followed by an insignificant increase.

When the diet contained equal parts of ham and egg protein (no. 6), the growth-promoting value was moderately higher than the computed value, but lower, although not significantly, than the value for eggs alone. When this diet was supplemented with cystine, the nutritive value was increased above that for eggs but not enough to show statistical significance.

The growth-promoting value of diet 8 containing 2 parts of ham and 1 part of egg protein was only slightly higher than the computed value, but the addition of cystine definitely increased the nutritive value of the protein above that for eggs.

The results of experiment no. 2 indicate only a moderate supplemental relationship between the protein in cured ham and that in uncooked, spray-dried eggs. The supplemental value of methionine for pork protein, and of cystine for mixtures of pork and egg protein, is clearly apparent.

In table 2 are shown the results of experiment no. 3 with cured ham, dried hard-boiled eggs, and with mixtures of these foods. A summary of the statistical comparisons shows the following:

Diet 1 with diets 2, 3, 4, 5, 6, 7, and 8 — very highly significant ($< .001$)
Diet 6 with diet 7 — highly significant ($< .01$)
Diet 6 with diets 2 and 5 — significant ($< .05$)
Diet 8 with diets 2, 3, 5, and 7 — highly significant ($< .01$)
Differences between all other diets — not significant.

The growth-promoting value of the protein in eggs was definitely higher than that for ham, but when ham was supplemented with methionine, the nutritive value of the protein was

TABLE 2

Supplemental relationship between the protein in cured ham and that in dried, hard-boiled eggs in 30-day tests with groups of 8 male rats (experiment no. 3).

SOURCE OF NITROGEN IN DIET	DIET NO.	GAIN IN WEIGHT		FEED CONSUMED	NITROGEN IN DIET	GAIN IN WEIGHT PER GRAM OF NITROGEN CONSUMED	
		Actual	Computed			Actual	Computed
		gm	*gm*	*gm*	%	*gm*	*gm*
Cured ham no. 4464	1	108	...	339	1.60	19.77	....
Cured ham no. 4464 + 0.2% methionine	2	125	...	327	1.62	23.44	....
Dried eggs no. 4466	3	129	...	344	1.60	23.40	....
Dried eggs no. 4468 + 0.2% methionine	4	135	...	336	1.62	24.86	....
Cured ham no. 4464, 0.8% N + dried eggs no. 4466, 0.8% N	5	139	119	373	1.60	23.27	21.59
Cured ham no. 4464, 0.8% N + dried eggs no. 4466, 0.8% N + 0.2% methionine	6	142	119	354	1.62	24.72	21.59
Cured ham no. 4464, 1.067% N + dried eggs no. 4466, 0.533% N	7	139	115	375	1.60	23.09	20.98
Cured ham no. 4464, 1.067% N + dried eggs no. 4466, 0.533% N + 0.2% methionine	8	140	115	342	1.62	25.24	20.98

practically the same as that of eggs. When eggs were supplemented with methionine (diet 4), there was an apparent slight increase in growth-promoting value. A mixture of equal parts of ham and egg protein had practically the same growth-promoting value as eggs alone, and a higher value than the computed one. When this mixture was supplemented with methionine, the nutritive value was materially increased. Diet 7 containing 2 parts of cured ham protein and 1 part of cooked egg protein induced nearly as efficient growth as eggs alone, and much better growth than the computed value. When this diet was supplemented with methionine (diet 8), the growth-promoting value was significantly increased.

The results of experiment no. 3 indicate a definite supplemental relationship between the protein in hard-boiled eggs and that in cured ham, but the growth-promoting value was further improved by the addition of methionine. The nutritive value of the protein in cured ham was increased to that of cooked eggs by the addition of methionine.

In table 3 are shown the results of experiment no. 4 concerning the supplemental relationship between the protein in dried, hard-boiled egg white and that in fresh ham. The statistical comparisons gave the following:

Diet 1 with diets 2, 3, 4, 5, and 6 — very highly significant ($< .001$)
Diet 6 with diets 3 and 5 — very highly significant ($< .001$)
Diet 6 with diet 4 — highly significant ($< .01$)
Differences between all other diets — not significant.

The growth-promoting value of egg white protein in diet 3 was materially higher than that of fresh ham, but when the latter was supplemented with cystine, the nutritive value was increased above that for egg white alone although not enough to be significant (diet 2 versus diet 3). A mixture of equal parts of ham and egg white protein in diet 4 did not give a significant increase in nutritive value over that of egg white alone. Similar results were obtained with a mixture of 2 parts of ham and 1 part of egg white protein. However, when the latter mixture was supplemented with cystine (diet 6), the growth-promoting value was definitely increased.

None of the rats in experiment no. 4 with cooked egg white, or in the previous experiments with either raw or cooked eggs, showed any symptoms of biotin deficiency. At the end of the 30-day test with the rats on the diet containing 10% of egg white protein, 2 rats were continued on the same diet for 30 days longer during which period each rat was fed 6 μg of biotin weekly. No increase in rate of growth was observed.

TABLE 3

Supplemental relationship between the protein in fresh ham and that in dried, hard-boiled egg white in 30-day tests with groups of 8 male rats (experiment no. 4).

SOURCE OF NITROGEN IN DIET	DIET NO.	GAIN IN WEIGHT		FEED CONSUMED	NITROGEN IN DIET	GAIN IN WEIGHT PER GRAM OF NITROGEN CONSUMED	
		Actual	Computed			Actual	Computed
		gm	*gm*	*gm*	%	*gm*	*gm*
Fresh ham no. 4471	1	111	...	332	1.60	20.83	....
Fresh ham no. 4471 + 0.4% cystine	2	141	...	352	1.65	24.26	....
Dried egg white no. 4470	3	126	...	343	1.60	23.00	....
Fresh ham no. 4471, 0.8% N + dried egg white no. 4470, 0.8% N	4	128	119	342	1.60	23.36	21.97
Fresh ham no. 4471, 1.067% N + dried egg white no. 4470, 0.533% N	5	126	...	338	1.60	23.17	21.56
Fresh ham no. 4471, 1.067% N + dried egg white no. 4470, 0.533% N + 0.2% cystine	6	144	...	347	1.62	25.55	21.56

The results of experiment no. 4 indicate a definite supplemental relationship between the protein in fresh ham and that in cooked egg white, but the growth-promoting value of a mixture was increased above that for egg white alone by the addition of cystine.

SUMMARY

The supplemental relationship between the protein in pork and that in eggs, and the effectiveness of cystine and meth-

ionine in correcting the deficiency in pork, were determined by experiments with young male albino rats with the following results:

The protein in both fresh and cured hams contained insufficient cystine and methionine for optimum growth in rats when the diets contained 10% of protein; but when pork protein was supplemented with these amino acids, the growth-promoting value was equal to that of protein in eggs.

There was found to be a moderate supplemental relationship between the protein in ham and that in both whole eggs and egg whites, but this relationship was materially improved by the addition of cystine or methionine. Mixtures of equal parts of pork and whole egg protein had approximately the same growth-promoting value as egg protein alone, and similar results were obtained with mixtures of equal parts of pork and egg white protein.

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EFFECT OF ENVIRONMENT ON GROWTH AND FEED AND WATER CONSUMPTION OF CHICKENS

I. THE EFFECT OF TEMPERATURE OF ENVIRONMENT DURING THE FIRST NINE DAYS AFTER HATCH

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FIVE FIGURES

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INTRODUCTION

In recent studies with the respiration calorimeter, Barott and Pringle ('46) noted that temperature of environment had an important effect on the energy and gaseous metabolism of chickens. Baby chicks at an environmental temperature of 70°F. eliminated 2.35 times as much heat as at 95°F., 5-week-old chickens 1.85 times as much at 45°F. as at 95°F., etc.

Homoiotherms consume more food at low temperatures than at high ones. This must be true because at the lower temperatures more food must be metabolized to regulate body temperature. However, the capacity of an animal to consume and metabolize foodstuffs is limited; therefore, one would expect that there would be a minimum temperature below which an animal would perish because it could not metabolize enough food for maintenance. At very high temperatures an animal does not grow well, probably because of insufficient food consumption, and above a certain point perishes because it is not

able to cool itself sufficiently to maintain body temperature. Between the minimum and maximum for existence there must be a temperature at which the efficiency, i.e., the growth increment divided by the food intake, is a maximum.

Because there are few data on the effect of temperature and other physical factors of environment on growth and feed and water consumption of chickens, an investigation of this subject was undertaken at the Agricultural Research Center. Since such an investigation must be extensive to cover the life cycle of the chicken and study the effect of each factor (temperature, humidity, light, etc.) separately for all ages, it was decided to publish the results as they were obtained. The temperature factor is undoubtedly the predominant influence; therefore, its effect was studied first and this paper discusses the results obtained with various temperatures, chickens from time of hatch to 9 days of age being used.

A search of the literature reveals a dearth of information on growth and feed consumption for the first few days after hatch, and as far as could be determined no values have been published on the day-by-day efficiency of feed utilization during the first week.

The economic importance of determining the optimum conditions for brooding can be easily understood from the following: In 1945 there were hatched in the United States alone 1,600,000,000 chicks with a monetary value of approximately $180,000,000. A study by the Ohio Poultry Improvement Association ('43–'44), of 8,317 groups totalling 2,400,000 chickens showed a mortality of 4% during the first 3 weeks after hatch. These were selected groups from large commercial hatcheries. Over the country as a whole, the loss would undoubtedly run considerably higher. If, by increased knowledge of proper environmental conditions for brooding, even one-fourth of this loss could be prevented, and investigation indicates that it could be, a saving of approximately $2,000,000 per annum would be affected.

APPARATUS

For the proper conduct of such an investigation, the chickens under observation must be housed in such a manner that all the physical factors relative to environment are under accurate control at all times and that any 1 factor may be varied at will while the other factors remain unchanged.

A room of sufficient size to accommodate the necessary equipment was, therefore, selected. This room is equipped with air-conditioning apparatus to control both the temperature and humidity. A description of the control apparatus for this room has been published by Barott ('39).

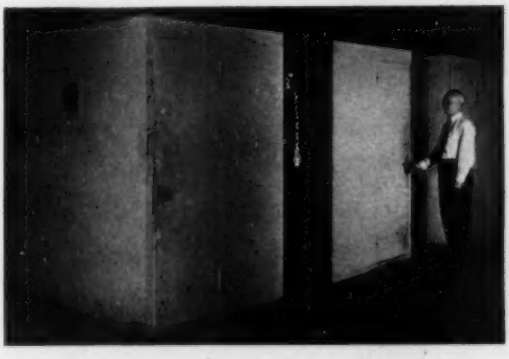

Fig. 1 Exterior of the 3 houses used in the experiments.

Three small houses (fig. 1) were built within this room. Each is 5 feet by 6 feet by 7 feet high. The walls, floor and ceiling are constructed of compressed cork board 2 inches thick, covered on either side with ¼-inch wall board which is painted white. Each house is fitted with apparatus for the accurate control of all the physical factors to be investigated.

Figure 2 shows a view of the interior of one of these houses. For temperature control, an electric heater coil (fig. 2a) is supported from the frame of a fan (fig. 2b) in such a manner that the air from the fan passes over the turns of the coil. The power supply to this heater is controlled by a bi-metallic

thermostat (fig. 2c) which may be set for any temperature between 0° and 200°F. and has a sensitivity of ± 0.35°F. The fan is in motion at all times whether the heater is on or off, in order to keep the air in the house well stirred so that there will be little temperature or humidity gradient.

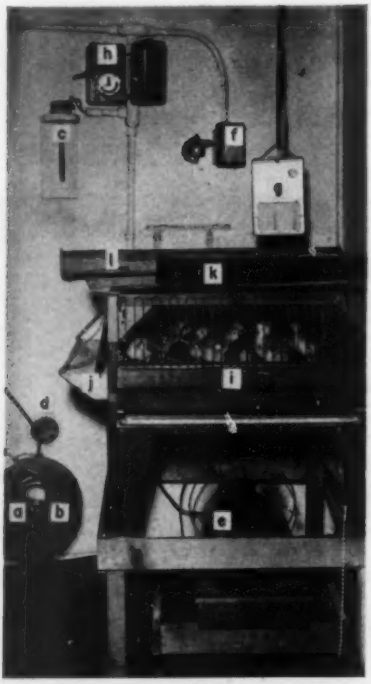

Fig. 2 Interior of one of the houses with controlled environment.

For ventilation purposes there are 2 vents in the ceiling and a vent with a damper in the rear wall (fig. 2d). By adjusting these apertures as much fresh air as desired may be admitted.

The humidification of the air is accomplished as follows: A light copper wheel (fig. 2e), 9 inches in diameter with 64 vanes, each 3 inches by 3 inches, is mounted on ball bearings in such a manner that as the wheel rotates the vanes dip into

a pan of water which is covered except for the opening for the wheel. This wheel is made to rotate by the air from a fan which is placed at the rear of the wheel. As the dampened vanes of the rotating wheel come within the air stream from the fan, sufficient water is vaporized and carried away in the air stream to bring the humidity inside the house to any predetermined level. Humidity control is by the humidistat (fig. 2f), which controls the power supply to the fan. When the humidity reaches the desired level the humidistat opens the fan motor circuit, causing the fan to stop and, therefore, also the wheel for humidification. As soon as the humidity drops slightly, the humidistat again starts the fan and thus the paddle wheel. By this means the humidity is always kept at the desired level.

A continuous record of temperature and humidity was kept on a chart of a Friez Recorder (fig. 2g) suspended directly over each cage. The recording apparatus was calibrated before the experiment started. The temperature graph was checked daily by a calibrated mercury thermometer and the humidity by an aspirating psychrometer.

A white fluorescent lamp, 4 feet long, of 40 watt capacity, attached to the ceiling directly over the cages, was used in each of the houses. Any type or color of light can be used by changing the bulb or, if necessary, the fixture. The light periods (of which there were 3 or 4) were controlled by a time clock (fig. 2h). The total duration of these periods was 12 hours out of each 24 hours.

PROCEDURE

The chickens used were from selected flocks of Rhode Island Reds. The breeding birds were maintained and the eggs hatched at the Agricultural Research Center. The chicks were removed from the hatcher when the average age was about 12 hours. They were not sexed but it developed that they were fairly evenly divided between the 2 sexes. A total of 300 to 400 chicks were provided for each test from which 90 of as nearly identical weight as possible were selected. The

average weight of all the chicks used was 40.5 gm, with a mean deviation in weight for the lot of approximately 1 gm. After selection, the chicks were leg banded and divided into 3 lots of 30 each. One group of 30 chicks was placed in each of the 3 houses in which the environmental conditions for the experiment had already been established. The chicks were brooded without a hover; consequently, they were at all times in an atmosphere of uniform temperature and humidity.

The following routine was observed at the same time each day. The chicks were weighed individually and as a unit and placed in the brooder. The water troughs (fig. 2i) were scrubbed and fresh water poured into them. Enough feed was added to the feed troughs (fig. 2j) to bring the feed in them up to the desired level. The troughs were weighed after these additions and the observed weights recorded as the initial weights of a 24-hour period. At the end of 24 hours, the chicks were removed from the brooder and weighed. The difference between this weight and the weight at the start of the period gave the gain in weight for that period. The feed and water troughs were also weighed at the end of 24 hours.

The difference between the initial and final weights equalled the amount of feed eaten and water drunk after correction for evaporation of water from the water trough and evaporation or absorption of water vapor from the atmosphere by the feed troughs. Correction factors for this gain or loss of water were obtained by using 2 check troughs (fig. 2k and 2l) identical in shape and size to those used for the chicks. These troughs were provided with the same amount of feed and water as the regular troughs but were placed out of reach of the chicks. The change in weight of these troughs was obtained each day and applied as a correction factor to the amount of water drunk and feed eaten. The diet (with the addition of 1 mg of synthetic riboflavin per kilogram of feed) fed to the chicks throughout the experiments had the following percentage composition: ground yellow corn, 37.575; ground wheat, 20.00; ground oats, 10.00; alfalfa leaf meal, 3.00; soybean meal, 20.00; sardine meal, 5.00; dried skim

milk, 2.00; ground limestone, 1.00; steamed bonemeal, .90; salt (96% NaCl; 4% mn.So_4), .50, and vitamin D powder (2000 μg/gm), .025.

The first experiment was started May 22, 1944, and 72 experiments have been made with chicks from time of hatch to 9 days of age. As the environment in each small house was controlled independently of the other houses, it was possible to conduct 3 experiments simultaneously. Thirty chicks were placed in each house during an experiment; consequently the work herein reported comprises data involving the use of 2,160 chicks. Mortality was practically nil. Occasionally a chick was caught in the wire floor and injured so that it had to be destroyed, but no deaths were due to infections or disease and none to environment.

When the analysis of the results was started, the question arose as to how to obtain the true gain in weight from day to day. Before hatching, the chick takes the yolk of the egg into the body cavity to be used as food material during the first few days after hatching; thus for several days the system contains decreasing amounts of this substance. When the chick starts to eat, undigested and unassimilated feed accumulates in the digestive tract. This feed and yolk are not properly a part of the chick until they are metabolized and utilized for body building. Therefore, it was decided to eliminate these factors and use the true increase in weight as a basis for analysis of the effect of temperature of environment.

To determine the quantity of extraneous matter, 30 chicks were dissected each day from the first day after hatching to the tenth day. They were dissected after a dark period (consequently after a period of no feeding) so that the crop was invariably empty. All weights used in these experiments were taken after a dark period of 4 hours so that the amount of feed in the system of the chick was comparable to that found by this dissection. The yolk sac and yolk and the contents of gizzard and of the intestinal tract of each chick were carefully isolated and weighed. A mean value for each of these quantities was taken for each day and the total amount was

used as a correction factor to the weight of the chick. In other words, the observed weight, minus foodstuffs within the body, gave the true weight for the purpose of analysis.

RESULTS AND DISCUSSION

Figure 3 shows the mean weight each day of (1) yolk sac and contained yolk, (2) gizzard contents and (3) intestinal contents. Curve 4 shows the sum of these 3 quantities and it was from this curve that the values were obtained which were subtracted from the observed weight of the chick each day.

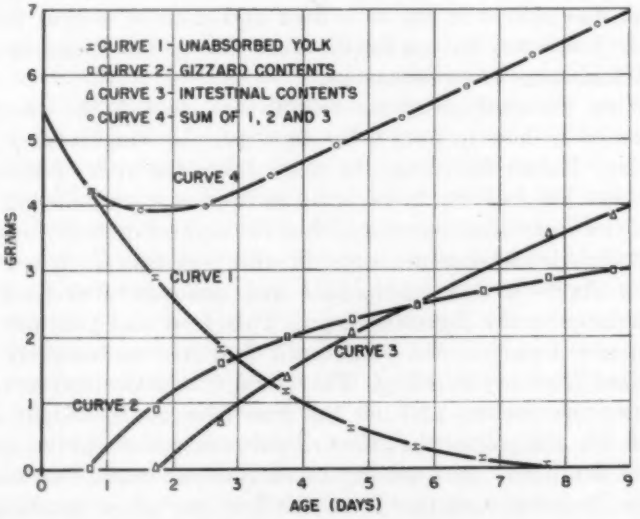

Fig. 3 Correction applied to chick weight for extraneous matter in system.

The difference in these corrected weights gave the true gain in weight from day to day.

Little is known about the optimal environmental temperature for brooding. United States Department of Agriculture Farmers Bulletin No. 1538 states that it is the custom to start brooders at about 95°F., and that in those with small hovers the temperature is reduced 3.5° to 5.0°F. weekly down to 70°F.

The heat is ordinarily cut off after the chick is well feathered. Electrically heated battery brooders are usually operated in a room where the temperature is maintained at from 60° to 70°F. Of course, this does not give much information as to optimal brooding temperature, for the chick can change position from the hover to the cooler environs at will. Whether the chick grows better and is more sturdy when brooded at a uniform temperature or as ordinarily with a hover is one of the questions which will be studied. All that can be said at present is that there was a mortality of less than 1% the first 9 days when the chicks were brooded without a hover, the chicks were well feathered and the growth was satisfactory.

In the experiments herewith reported the temperature ranged from 96° to 85°F. for the first day after hatch and from 92° to 80° for the ninth day after hatch.

The humidity was kept between 60 and 70% in all experiments and the feeding time was approximately 12 hours out of each 24-hour day. The total feeding time was divided into 3 or 4 periods.

The data obtained for each experiment were computed for (1) daily gain in weight in grams and in percentage increase over the original weight, (2) grams of feed consumed per chick per day, (3) grams of water consumed per chick per day.

From comparative analyses of the results obtained in all the experiments which were conducted at any given temperature, the percentage of gain in weight of the chicks for 9 days was obtained. The results for each temperature were plotted and curve 1, figure 4, constructed through the plotted points. The numeral at each point designates the number of experiments used to obtain that point, e.g., a point with the figure 11 designates 11 experiments of 30 chicks each or a mean for 330 chicks. The point is plotted at the temperature which is a mean for that group. The range in temperature for each group can be determined from table 1.

Curve 1, figure 4, shows a very definite variation in growth rate due to temperature. The maximum growth was obtained when the mean temperature was 91°F. and equalled a 59%

increase over the original weight in 9 days. This occurred when the chicks were kept in an environmental temperature of 94°–95°F. the first day and thereafter the temperature was decreased uniformly to 88°F. on the ninth day. The gain in weight was less at other temperatures studied and became

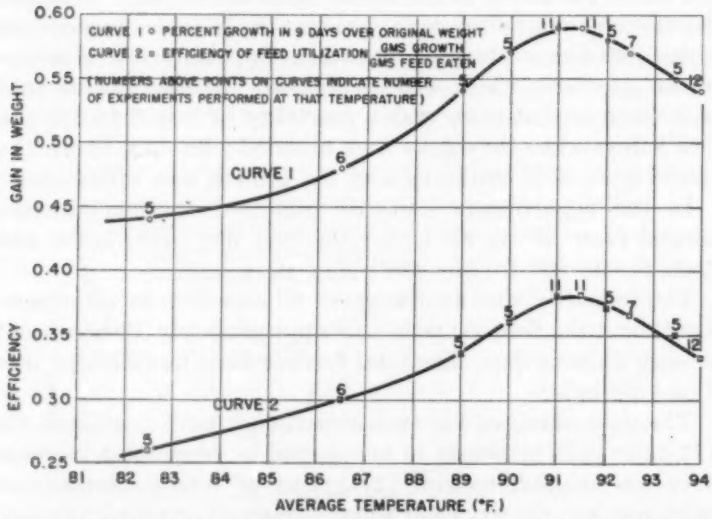
CURVE 1 = PERCENT GROWTH IN 9 DAYS OVER ORIGINAL WEIGHT
CURVE 2 = EFFICIENCY OF FEED UTILIZATION GMS GROWTH / GMS FEED EATEN
(NUMBERS ABOVE POINTS ON CURVES INDICATE NUMBER OF EXPERIMENTS PERFORMED AT THAT TEMPERATURE)
GAIN IN WEIGHT
EFFICIENCY
CURVE 1
CURVE 2
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
81 82 83 84 85 86 87 88 89 90 91 92 93 94
AVERAGE TEMPERATURE (°F.)

Fig. 4 Effect of temperature of environment on growth and efficiency of feed utilization during first 9 days of age.

TABLE 1

Number of experiments at temperatures studied.

NUMBER OF EXPERIMENTS	TEMPERATURE 1ST DAY (°F.)	TEMPERATURE 9TH DAY (°F.)
12	96	92
5	95	91
7	94	91
5	96	88
11	95	88
11	94	88
5	92	88
5	93	84
6	90	83
5	85	80

less and less the farther the deviation below or above the temperature where maximum growth occurred, until at a temperature of 82.5°F. (initial temperature 85°F., final temperature 80°F.), the increase in weight was only 44%.

Two experiments were performed with a constant temperature throughout the 9 days, 1 at 93°F., and 1 at 91°F. At 93°F. a 54% increase in weight over the 9 days was noted; at 91°F. a 55% increase. Since in each of these experiments the growth in 9 days was less than when the temperature was decreased day by day it was concluded that it was less satisfactory to maintain a constant temperature.

Kleiber and Dougherty ('33) published the results of a well-conducted experiment on the effect of environmental temperature on growth and feed consumption between 5 and 15 days of age. They found that the lower the temperature between 21° and 40°C. (69.8° and 104°F.) the better the growth. This was not true in our experiments with chicks between time of hatch and 9 days of age. However, it might be expected that younger chickens would need a higher temperature for satisfactory growth.

The *ad libitum* feed consumption per chick (a surplus of feed was before the chicks at all times so they could eat as much as they desired) was practically constant in amount between the temperatures of 82° and 94°F. Therefore, curve 2, figure 4, which shows the efficiency of the chick in feed utilization (grams gain in weight divided by the grams of feed eaten), has practically the same form as curve 1. At 91°F. the gain in weight equalled approximately 0.38 gm per gm of feed eaten. As the temperature was lowered the rate of growth became less and less so that at an average temperature of 82°F. the efficiency had dropped to 0.26 gm of growth per gm of feed. The efficiency also became increasingly less as the temperature was raised above 91°F. until at 94°F., it was 0.33 gm of growth per gm of feed eaten.

After the range of temperature was determined within which the growth of the chicks was maximum, results from the 22 experiments (660 chicks) conducted within this range

were analyzed to obtain the values shown by curves 1 to 4, inclusive, in figure 5.

Curve 1, showing the increase in weight each day in grams, seems to be nearly lineal for the first 5 days; thereafter the rate appears to accelerate.

Curve 2 showing percentage increase over original weight, is the typical growth curve for chicks. Growth starts slowly for the first days after hatch and accelerates each day until

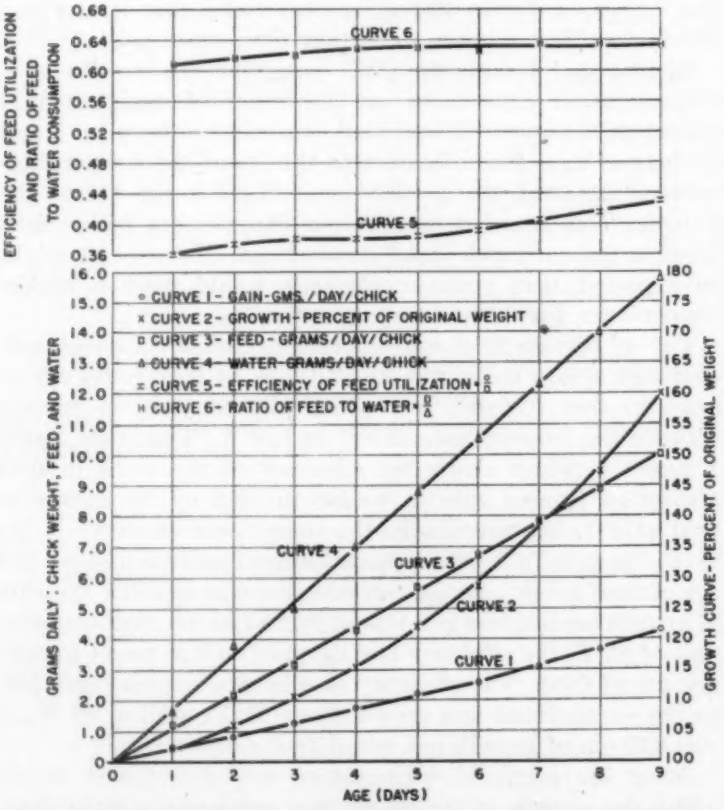
EFFICIENCY OF FEED UTILIZATION AND RATIO OF FEED TO WATER CONSUMPTION
CURVE 6
CURVE 5
o CURVE 1 - GAIN-GMS./DAY/CHICK
x CURVE 2 - GROWTH - PERCENT OF ORIGINAL WEIGHT
□ CURVE 3 - FEED - GRAMS/DAY/CHICK
△ CURVE 4 - WATER-GRAMS/DAY/CHICK
= CURVE 5 - EFFICIENCY OF FEED UTILIZATION
H CURVE 6 - RATIO OF FEED TO WATER
GRAMS DAILY : CHICK WEIGHT, FEED, AND WATER
GROWTH CURVE - PERCENT OF ORIGINAL WEIGHT
AGE (DAYS)

Fig. 5 Growth and feed and water consumption of chickens from hatch to 9 days of age.

the growth between the eighth and ninth days is 6 times greater than that between the start and the first day. The growth between the eighth and ninth days was over 10% of the weight on the eighth day.

Curves 3 and 4, showing feed and water consumption, appear to be lineal, the amount consumed each day being approximately 1.0 gm of feed per chick more than was consumed the previous day and 1.7 gm of water per chick more than the previous day.

From curves 1 and 3 (fig. 5), it was possible to compute the efficiency of the chick in the utilization of foodstuffs. The daily gain in weight in grams (from curve 1) divided by the daily feed consumption (from curve 3) equals the efficiency. Results obtained in this manner are shown in curve 5, drawn through the plotted points. On the first day the efficiency was found to be 0.36. It rose to 0.38 on the third day, remained practically constant at that figure on the fourth and fifth days and rose to 0.43 on the ninth day.

Kleiber and Dougherty ('33) found an efficiency of feed utilization, based on dry weight of feed, of 0.375 at 32°C. (89.6°F.) for chicks between 5 and 15 days of age. In the investigation herein reported with chicks between time of hatch and 9 days of age an efficiency of 0.35 was found at this same temperature. The feed given to these chicks contained 10% of moisture. When the feed was calculated on a dry weight basis, the efficiency of the chicks at 32°C. (89.6°F.) was 0.39.

Hammond et al. ('38) indicated the efficiency of feed utilization as a straight line with a maximum value at hatch and decreasing to zero when growth ceases. This conclusion was based on weekly weights of chickens. The efficiency reported herein is least at hatch and increases gradually for the first 9 days. It will ultimately become stable and then begin to decrease because the efficiency when growth ceases is zero.

Curve 6 (fig. 5), shows the relation between feed consumed and water drunk, i.e., grams of feed consumed per chick per day, divided by the grams of water drunk per chick per day.

This curve starts at 0.61, rises to 0.63 on the fourth day and remains constant at this figure to the ninth day. On the ninth day after hatch, the chick consumed approximately 2.35 gm of feed to increase its weight 1 gm and drank approximately 1.6 gm of water for each gm of feed eaten.

SUMMARY

Chicks were housed in controlled environment without a hover for the first 9 days after hatch in order to determine the optimum temperature for growth and efficiency of feed utilization. Thirty chicks were used in each experiment and a total of 72 experiments were performed. Mortality for all experiments was practically nil. Relative humidity was kept between 60 and 70% in all experiments. The chicks were allowed 12 hours ad libitum feeding time, divided into 3 or 4 periods with an equal dark period between each feeding period.

The temperature was varied with different lots of chicks, from a mean temperature of 94°F. over the 9 days (96° for the first day and gradually reduced to 92°F. the ninth day) to a mean temperature of 82.5°F. over the 9 days (85°F. the first day and gradually reduced to 80°F. the ninth day).

Maximum growth and efficiency of feed utilization were noted when the temperature was 94°–95°F. the first day dropping uniformly to 88°F. on the ninth day. The growth under these conditions equalled a 59% increase over the original weight in 9 days. (All weights of chicks were obtained 4 hours after the last feeding period, and therefore there was no feed in the crop.) The mean efficiency of feed utilization over the 9 days was 0.38. Both growth and efficiency became less as the temperature varied from this range: the greater the deviation, the greater the difference, until at 82.5°F. (85° to 80°F.) the increase in weight was only 44% and the efficiency 0.26. At 94°F. (96°–92°F.) the increase in weight was 54% and the efficiency 0.33. The amount of feed consumed increased approximately 1 gm per day per chick. On the ninth day after hatch the chick consumed approximately 2.35 gm of feed for

every gm increase in weight and drank approximately 1.6 gm of water for each gm of feed consumed.

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RIBOFLAVIN EXCRETIONS AND TEST DOSE RETURNS OF YOUNG WOMEN DURING PERIODS OF POSITIVE AND NEGATIVE NITROGEN BALANCE

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An inverse relationship between the protein intake and the urinary excretion of riboflavin in dogs and rats was demonstrated by the studies of Sarett and associates ('42, '43). They found that a decrease in the protein content of the diet, associated with a negative nitrogen balance produced an increase in the urinary excretion of riboflavin; an increase in the protein content of the diet, associated with a positive nitrogen balance, produced a decrease in the urinary riboflavin excretion. That such a relationship also existed in children was indicated in a study carried out in our laboratory (Oldham et al., '44). When 2 pre-school children were on low-protein intakes during the course of acute upper respiratory infections, their urinary riboflavin excretions increased; when they returned to normal protein intakes, their riboflavin excretions decreased despite the fact that their riboflavin intakes had been maintained at a constant level. Both the reduced protein intakes and the elevations in body temperature which accompany infections usually tend to produce negative nitrogen balances. Therefore, although the nitrogen balances were not determined, it seemed that, in all probability, they were negative.

The 3-fold purpose of the present study was to note the effect of high- and low-protein intakes on (a) the nitrogen

[1] These data were presented by Elizabeth Lounds in partial fulfillment of the requirements for the degree of Master of Science at the University of Chicago, June, 1946.

balances, (b) the excretions of riboflavin and (c) the riboflavin test dose returns of young women.

EXPERIMENTAL

The subjects were 3 young women, 26 to 27 years of age, whose physical examinations showed no abnormalities. Prior to the study, the dietary histories of subjects C and E showed apparently adequate intakes of all essential nutrients. That of subject L indicated an apparently inadequate intake; both her protein and her riboflavin intake were judged to be less than adequate according to present day standards.

After a 5-day preliminary period, the subjects were maintained on weighed diets for 3 periods of 10 days each. The protein intakes were low during the first period, high during the second and low during the third. The riboflavin intakes were relatively constant throughout the study and were somewhat lower than the recommended allowances for women.

A typical day's diet on both the low- and the high-protein intakes is shown in table 1. A variety of foods was served daily and the servings were of average size in most cases. In addition, as much day-to-day variation was allowed in the kinds of fruits and vegetables served as was possible without significantly altering the daily protein and riboflavin intakes.

The diets were planned to meet the recommended daily allowances of the Food and Nutrition Board of the National Research Council ('45) for all nutrients except protein and riboflavin. Daily supplements of 700–900 mg of calcium as dicalcium phosphate wafers, 0.5 mg of thiamine [2] and 10 mg of niacin were given in the periods of low-protein intake (I and III) to complete the allowances and to more nearly approximate the daily intakes of period II.

The average daily intakes of different nutrients were calculated for 5 days during each period and are shown in table 2. Tables of food values compiled by Bowes and Church ('44)

[2] We wish to express our appreciation to Merck and Company for furnishing the synthetic vitamins used in the study.

TABLE 1

Typical menus on low and high protein diets.

BREAKFAST	gm	LUNCH	gm	DINNER	gm
		Low-protein diet			
Orange juice	100	Baked potato	100	Ham	40
Blueberries	75	Asparagus	100	Yams	100
Rice flakes	20	Carrots	100	Cauliflower	75
Bread	15	Prunes	50	Head lettuce	50
Cream	100	Lettuce	20	French dressing	10
		Mayonnaise	10	Crushed pineapple	100
		Jello	100	Bread	15
		Bread	20	Lemon juice	30
		Grapefruit juice	150		
		High-protein diet			
Grapes	100	Roast lamb	100	Baked trout	150
Cornflakes	25	Green beans	50	Corn	100
Bacon	12	Squash	100	Cabbage	50
Bread	45	Celery hearts	50	French dressing	10
Cream	50	Applesauce	100	Orange	100
		Bread	45	Bread	45
		Peanut butter	20	Photolyzed non-fat milk solids [1]	50
		Punch:			
		Grape juice	100		
		Lemon juice	50		
		Gelatin	20		

Ad libitum on both diets: sugar, butter, jelly and coffee.

[1] Served with sugar and vanilla as a milk-shake.

TABLE 2

Calculated average daily intakes of different nutrients.

PERIOD	CAL.	PROTEIN	FAT	CARBOHYDRATE	Ca	P	Fe	VIT. A	THIAMINE	RIBOFLAVIN	NIACIN	VIT. C
		gm	gm	gm	gm	gm		I.U.	mg	mg	mg	mg
I	1508	32	60	210	1.3	2.1	9	10,390	1.4	0.9	19	162
II	2133	110	81	241	0.8	1.4	12	3,360	1.2	1.1	23	122
III	2024	28	80	298	1.1	1.7	10	13,590	1.5	0.9	18	206
Recommended allowance	2100	60			0.8		12	5,000	1.1	1.5	11	70

and by Taylor ('42) were used. That the caloric intake was undesirably low in period I was verified by weight losses in all of the subjects; this condition was corrected in periods II and III during which time usual weights were regained and maintained. The amount of iron was slightly lower than that recommended, but it seemed highly improbable that this would influence the results in a study of such short duration. Therefore, no iron supplement was given. The vitamin A intake was low in period II, but the average intake for all periods was well over the recommended allowance.

Individual food composites, consisting of one-fifth of the amount of each food eaten with the exception of bread, dry cereals and coffee, were made for 5-day periods. The bread, dry cereals and coffee were analyzed separately. Butter and cane sugar were not analyzed.

Test doses of 20 μg of riboflavin per kilogram of body weight were given immediately before breakfast at the beginning of the study and at the end of each experimental period; this was followed by a 4-hour urine collection. On the previous day, after an identical breakfast, a 4-hour control urine specimen was collected. The breakfasts on these days were essentially the same throughout the study, although the diet as a whole was a continuation of the diet in the preceding period. The test dose returns were calculated by subtracting the amount of riboflavin excreted in the control sample from that excreted after the test dose.

Complete collections of both urine and feces were made throughout each experimental period. Twenty-four urine specimens were collected daily. Each sample was immediately preserved with hydrochloric acid and toluene and then refrigerated. Five-day fecal collections, marked with carmine, were homogenized with 95% alcohol, acidified with hydrochloric acid to a pH of 1.0 and refrigerated.

The daily urine specimens and the 5-day food and fecal composites were analyzed for nitrogen by the Kjeldahl method and for riboflavin by the microbiological method of Snell and Strong ('39) as modified by Strong and Carpenter ('42).

Daily nitrogen balances were calculated by subtracting the daily urinary excretions and average daily fecal excretions from the average daily intakes.

RESULTS AND DISCUSSION

Riboflavin excretions and nitrogen balances

The average daily riboflavin and nitrogen intakes, the corresponding fecal excretions, the daily urinary excretions and the daily nitrogen balances of the individual subjects for the first and last half of each period are shown in table 3.

On daily nitrogen intakes of approximately 5 gm in period I, subject C was in negative nitrogen balance for 9 out of 10 days and E for the entire 10 days. The balances were decidedly negative during the first part of the period. Later they were only slightly negative and by the eighth day they were in approximate equilibrium. The corresponding daily riboflavin excretions were 600 μg or more (approximately 60% of the intake) when the nitrogen balances were strongly negative and from 200 to 350 μg (approximately 30% of the intake) when the nitrogen balances were only slightly negative.

In period II, on nitrogen intakes of 18 and 19 gm both subjects were in positive nitrogen balance for the entire 10 days. In both cases, the greatest storage occurred during the first part of the period. Although the riboflavin intakes were somewhat higher in this period than in period I (1285 and 1450 μg as compared to 950 μg), the daily riboflavin excretions immediately dropped to about 100 μg (approximately 7% of the intake).

In period III, when both the nitrogen and the riboflavin intakes of subjects C and E were comparable to those in period I, the nitrogen balances were again decidedly negative during the first part of the period and approached equilibrium in the last part. The daily riboflavin excretions were decidedly higher than those of period II. They represented almost 40% of the intake when the nitrogen balances were strongly negative and about 20% of the intake as nitrogen equilibrium was approached.

TABLE 3

Intakes and excretions of nitrogen and riboflavin.

PERIOD	NITROGEN				RIBOFLAVIN		
	Intake	Urine	Feces	Balance	Intake	Urinary excretion	Fecal excretion
	gm/av. day	*gm/day*	*gm/av. day*	*gm*	*μg/av. day*	*μg/day*	*μg/av. day*
				Subject C			
Ia	4.87	7.27, 8.10, 5.70, 6.31, 3.74	0.74	— 3.14, — 3.97, — 1.57, — 2.18, + 0.39	972	738, 622, 694, 636, 226	422
Ib	4.76	4.64, 5.14, 4.93, 4.81, 4.45	0.61	— 0.49, — 0.99, — 0.78, — 0.66, — 0.30	893	249, 254, 344, 210, 193	318
IIa	17.74	7.98, 10.23, 11.78, 13.35, 12.84	0.88	+ 8.88, + 6.63, + 5.08, + 3.51, + 4.02	1237	158, 94, 101, 154, 73	442
IIb	17.68	14.03, 13.92, 10.02, 16.52, 16.29	0.87	+ 2.78, + 2.89, + 6.79, + 0.29, + 0.52	1322	93, 77, 103, 182, 114	430
IIIa	4.83	9.91, 6.11, 6.11, 5.58, 5.17	0.79	— 5.87, — 2.07, — 2.07, — 1.54, — 1.13	1016	494, 507, 250, 379, 317	601
IIIb	4.82	4.55, 4.69, 4.34, 3.98, 4.08	0.81	— 0.54, — 0.68, — 0.33, + 0.03, — 0.07	859	308, 279, 128, 124, 196	473
				Subject E			
Ia	5.20	7.22, 6.76, 5.91 5.85, 5.85	1.08	— 3.10, — 2.64, — 1.79, — 1.73, — 1.73	999	722, 695, 577, 615, 392	624
Ib	4.71	4.57, 4.72, 4.56, 4.17, 3.97	1.16	— 1.02, — 1.17, — 1.01, — 0.62, — 0.42	957	328, 242, 342, 339, 278	949
IIa	18.63	8.37, 11.96, 12.83, 15.58, 14.62	1.64	+ 8.62, + 5.03, + 4.16, + 1.41, + 2.37	1519	201, 103, 101, 149, 97	883
IIb	19.36	12.79, 15.83, 15.48, 17.35, 16.70	1.25	+ 5.32, + 2.28, + 2.63, + 0.76, + 1.41	1383	172, 101, 130, 126, 147	765
IIIa	4.98	9.67, 6.21, 5.16, 5.55, 5.14	1.51	— 6.20, — 3.74, — 1.69, — 2.08, — 1.67	945	316, 379, 234, 238, 263	877
IIIb	4.90	4.35, 4.77, 4.85, 4.36, 4.25	1.05	— 0.50, — 0.92, — 1.00, — 0.51, — 0.40	935	310, 215, 185, 131, 238	825
				Subject L			
Ia	5.85	4.51, 5.02, 4.78, 3.54, 4.02	1.18	+ 0.16, — 0.35, — 0.11, + 1.13, + 0.65	1060	391, 396, 358, 223, 220	894
Ib	5.19	3.77, 1.38, 3.45, 3.68, 5.55	1.35	+ 0.07, + 2.46, + 0.39, + 0.16, — 1.71	959	225, 61, 182, 183, 227	985
IIa	20.14	7.52, 6.36, 12.70, 7.64, 14.18	1.19	+ 11.43, + 12.59, + 6.25, + 11.31, + 4.77	1495	205, 101, 111, 76, 100	819
IIb	20.09	14.13, 6.37, 8.52, 10.99, 10.88	1.77	+ 4.19, + 11.95, + 9.80, + 7.33, + 7.44	1441	177, 91, 103, 154, 124	1314
IIIa	5.57	6.41, 6.86, 5.99, 4.42, 4.38	1.38	— 2.22, — 2.67, — 1.80, — 0.23, — 0.19	1025	287, 273, 270, 213, 211	1194
IIIb	5.23	3.26, 7.14, 3.81, 2.73, 3.94	1.31	+ 0.66, — 3.22, + 0.11, + 1.30, — 0.02	965	265, 182, 176, 130, 152	942

Although the nitrogen losses of both subjects were greater in period III than in period I, the riboflavin excretions were less. This can probably be explained on the basis of the diets prior to the study. Both subjects had regularly consumed a diet which included at least 1½ pints of milk daily as well as generous amounts of meat, eggs, vegetables and fruit. Their daily riboflavin intakes on such diets were obviously considerably more than 1000 μg. Therefore, they excreted relatively large amounts of riboflavin during the first few days as they adjusted to the lower intakes.

The results obtained on subject L show the same general pattern as those on subjects C and E, but at a different level. Her nitrogen balances were for the most part, slightly positive during period I on the low nitrogen intake. This indicated that the nitrogen content of her diet prior to the experiment had probably been quite similar to that of her diet in period I. Additional evidence of this was obtained from her dietary history which showed that her eating habits had been both irregular and erratic; for the most part she had subsisted on snacks taken at odd intervals rather than regular meals. Still further evidence of her depleted state is shown by the magnitude of her nitrogen balances in period II on the high-protein diet. She stored over twice as much nitrogen in that period as subjects C and E. Following these strongly positive balances, her balances were negative in period III, although to a lesser degree than those of subjects C and E. The daily riboflavin excretions of subject L followed the same general trend as those of subjects C and E, but also to a lesser degree.

Correlation coefficients,[2] calculated between the daily nitrogen balances and riboflavin excretions were found to be highly significant for all 3 subjects. They were — 0.69, — 0.57 and — 0.69 for subjects C, E and L, respectively.

This shows that in human subjects as in animals an inverse relationship exists between urinary riboflavin excretions and nitrogen balances. This relationship seems to hold, regardless

[2] Correlation coefficients were calculated according to the Product-Moment formula.

of whether the negative nitrogen balances are brought about by a low-protein diet or by other factors (Andrea et al., '46; Roderuck et al., '46). It also seems to be independent of small changes in body weight. In the present study, weight losses of 1 to 2½ kg occurred in all 3 subjects during period I and gains of approximately 1 kg were made by 2 of the subjects in period II. In period III, however, only 1 subject showed a change in weight.

It is not surprising that riboflavin and labile protein are closely related since the main storage organ of both is the liver (Luck, '36; Kosterlitz and Campbell, '45). Although the kidney also has a high concentration of riboflavin (Taylor et al., '42; Mitchell and Isbell, '42), Czaczkes and Guggenheim ('46) report that it is unable to store this vitamin.

That the riboflavin content of the livers of animals is dependent on protein intakes rather than on riboflavin intakes has been demonstrated in several laboratories (Sarett and Perlzweig '43; Unna et al., '44; Reisen et al., '46; Czaczkes and Guggenheim, '46; Wright and Skeggs, '46). The relatively high riboflavin excretions during periods of negative nitrogen balance may be the result of the inability of the liver to store riboflavin or the release of previously stored riboflavin or both. The findings of Czaczkes and Guggenheim ('46), however, indicate that the low riboflavin excretions during periods of positive nitrogen balance cannot be due to storage of the vitamin by the liver. These authors believe them to be due to an increased dietary requirement which in turn, results from a decreased intestinal synthesis since both the fecal riboflavin excretion and the number of viable bacteria in the feces of their animals on the high-protein diet were less than those of animals on a normal diet.

In the present study, however, no such decrease in fecal riboflavin occurred during the period of high-protein intake (table 3). Although these data cannot be interpreted as evidence that there was no change in the rate of intestinal synthesis, they do make it seem questionable and would lend support to the possibility that other factors may have been

operating, in the case of these subjects, to promote liver storage or to increase the requirement of the vitamin.

Riboflavin test dose returns

Since the amount of riboflavin excreted in the urine varied with the coexistent nitrogen metabolism, it would seem logical to expect the returns of riboflavin test doses to show a similar variation. Sarett et al. ('42) found such to be the case in dogs. In the present study the returns of subjects C and L were lower after periods of positive nitrogen balance or approximate equilibrium than after periods of negative nitrogen balance (table 4). Those of subject E, however, increased

TABLE 4

Per cent return of riboflavin test doses.

SUBJECT	PRE PERIOD			PERIOD I			PERIOD II			PERIOD III		
	Test dose	4-hour return [1]		Test dose	4-hour return [1]		Test dose	4-hour return [1]		Test dose	4-hour return [1]	
	μg	μg	%	μg	μg	%	μg	μg	%	μg	μg	%
C	1000	188	19	990	322	33	1001	224	22	986	366	37
E	1100	180	16	1089	207	19	1086	288	27	1087	332	31
L	1200	49	4	1238	54	4	1274	64	5	1256	113	9

[1] Corrected for the amount excreted during the 4-hour control period.

progressively throughout the study and showed no relationship to her nitrogen balances. As a result no definite conclusions can be drawn from these data as to the effect of nitrogen balances on test dose returns. However, since 2 of the subjects showed the same inverse relationship as was found by Sarett et al. ('42), on dogs, and since Czaczkes and Guggenheim ('46) reported that rats on a low-protein diet lacked the ability to retain riboflavin, it would seem advisable to give consideration to the nitrogen metabolism when interpreting the results of riboflavin test dose returns.

SUMMARY AND CONCLUSIONS

Three young women were maintained on weighed diets for 3 periods of 10 days each. The daily nitrogen intakes were

approximately 5 gm during the first period, 19 gm during the second and 5 gm during the third. The daily riboflavin intakes were approximately 1000 μg during the first period, 1200 to 1400 μg during the second and 1000 μg during the third. Urinary riboflavin and nitrogen excretions were determined daily; the amounts in foods and feces were determined on individual 5-day composites. Daily nitrogen balances were calculated by subtracting daily urinary excretions and average daily fecal excretions from average daily intakes.

The majority of the nitrogen balances were negative during the periods of low-nitrogen intake and all were positive during the period of high-nitrogen intake.

Daily riboflavin excretions varied inversely with the coexistent nitrogen balances. They represented from 40 to 60% of the riboflavin intake when the nitrogen balances were decidedly negative and approximately 7% of the intake when the nitrogen balances were strongly positive.

The returns of riboflavin test doses given at the end of each period showed the same inverse relationship with the nitrogen balances in 2 of the subjects.

Fecal riboflavin excretions did not appear to be influenced by the alterations in the composition of the diet and showed no relationship to the nitrogen balances.

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GROWTH AND LONGEVITY OF RATS FED OMNIVOROUS AND VEGETARIAN DIETS[1]

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THREE FIGURES

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Comparative studies of the effects of omnivorous and purely vegetarian diets on the growth and longevity of rats seem of interest as the increasing population of the world tends to compel the use of a larger proportion of foods of vegetable origin in the human diet and the view is widely held that a vegetarian diet promotes health and longevity. Slonaker ('12) determined the effect on rats of a diet which he called a strictly vegetarian diet but this diet included bread, cookies and other items which introduced small amounts of foods of animal origin, such as milk, butter and eggs. The growth of Slonaker's rats on the so-called strictly vegetarian diet was nevertheless impaired and they lived only about half as long (555 days) as control rats (1020 days) which were fed the same diet with the addition of meat and other foods of animal origin. Extensive comparative studies of the effects of purely vegetarian and omnivorous diets were made on rats by Wu and his associates (Wu and Wu, '28; Wu, Wan and Chen, '32; Chang, Wu and Chen, '41; and Chen, Chang and Luo, '41). The growth of rats on the best (economical) purely vegetarian diet that Wu and his associates devised was inferior to growth on their omnivorous diets but the vegetarians

[1] This study was aided by a grant from Swift and Co., Chicago.

were found to live as long or longer than the omnivorous rats (Chang, Wu and Chen). That is, the male vegetarians did not live significantly longer (540.1 days) than the omnivorous males (526.7 days) but the vegetarian females lived significantly longer (611.1 days) than the omnivorous females (526.9 days). The longer life span of the vegetarian females was attributed to their retarded growth but this does not explain why the vegetarian males failed to live significantly longer than the omnivorous males. The data of Chang, Wu and Chen show that their omnivorous females did not live longer than the omnivorous males and this is contrary to general findings. It therefore seems that, instead of the life span of the females having been lengthened by a vegetarian diet, the life span of the females on the omnivorous diets was somehow shortened. A further study of the effect of omnivorous and vegetarian diets on longevity and incidentally, on growth therefore appeared to be desirable.

METHODS

As indicated in a preceding paper (Carlson and Hoelzel, '46) we included the use of omnivorous diets and a purely vegetarian diet in a study of the effect of intermittent fasting on the longevity of rats. In that study, 45 Wistar rats (19 males and 26 females) were fed our basic omnivorous diet (diet 1) and 46 rats (21 males and 25 females) were fed the purely vegetarian diet (diet 4). Besides this 46 rats were fed diet 1 with 10% added cellulosic bulk-formers (diets 2 and 3) but the details concerning the effects of these and other bulky omnivorous diets will be reported in a separate paper. Diets 1 and 4 were described in the preceding paper and the composition is included again in table 1. Approximately equal numbers of the rats fed diet 1 and 4 were fed these diets ad libitum or were fasted 1 day in 4, 1 day in 3 or 1 day in 2. Littermates of 7 males and 18 females fed diet 1 were included in corresponding groups fed diet 4 and littermate rats were also included in the different groups on the same diet.

In a second longevity study on Wistar rats, diet 1 was again used as the basic omnivorous diet but, in place of diet 4, a vegetarian self-selection diet (diet 6, table 1) was used. Each item in diet 6 was presented in a separate container or in a separate compartment of a larger container. The peas and soy beans included in diet 6 were first soaked 18 hours in water. Most of the rats in this study were however fed diet 1

TABLE 1

Composition of diets.

Diet 1 — 61.5% dried "whole veal,"[1] 3% veal bone meal, 31% corn starch, 2% dried brewer's yeast, 1.5% inorganic salt mixture and 1% cod liver oil. Supplemented by lettuce ad libitum.

Diet 4 — 50% stone-ground whole wheat flour,[2] 10% peanut flour, 7% wheat gluten flour, 7% lima bean flour, 7% linseed meal, 7% corn gluten meal, 5% alfalfa leaf meal, 5% brewer's yeast, and 2% NaCl. Supplemented by lettuce ad libitum.

Diet 6 — Self-selection from corn (whole kernels), wheat (whole grain), pearled barley, rolled oats, sunflower seeds, peanuts, green peas, soy beans, defatted corn germ meal,[3] defatted wheat germ meal,[3] brewer's yeast, alfalfa leaf meal and NaCl. Supplemented by lettuce ad libitum.

Diet 6C — Diet 6 supplemented by celery cabbage as well as by lettuce ad libitum.

Diet 6CC — Diet 6 supplemented only by celery cabbage ad libitum.

[1] Specially prepared for us by Swift and Co., Chicago, and previously described (Carlson and Hoelzel, '46).

[2] Obtained from Elam Mills, Chicago.

[3] Obtained from VioBin Corp., Monticello, Ill.

with added cellulosic or other bulk-formers (diets 3, 5, 7, 8, 9, 10 and 11) but, as already stated, their specific effects will be the subject of a separate report. Other differences between the second and the first longevity study were as follows: The first aim was to use rats likely to be more uniform genetically than the rats used in our first study. This objective was presumably attained by using 18 litters obtained from 4 females and 2 males (father and son) instead of 17 litters obtained from 16 females mated with 6 males as in the first longevity study. No first litters were used and, to promote

pre-experimental nutritional uniformity, litters of more than 8 rats were reduced to 8, 7 or 6 rats. The 18 litters provided 115 rats (57 males and 58 females), including rats born in each of the 4 seasons. To make it possible to determine the influence of the different diets at the earliest possible ages, successive litters from the same mothers were raised from the time when they first began to eat on the different experimental diets. The mothers however were left with their litters until the litters were 35 days old but the mothers were fed supplementary food separately to keep them in as uniform a condition as practical for further breeding. The sires were kept on diet 1. Five litters, including 17 males and 16 females, were started on diet 1. Eleven litters were started on diet 1 with 10% added bulk-formers (diets 3, 5, 9 and 10). Only 2 litters, including 6 males and 7 females, were started on diet 6 before a deterioration of some of the rats on this diet suggested that the starting of more rats on this diet was not warranted. The original intention was to keep about half of the rats on the same diet throughout life and to transfer the other half to 1 of the other diets when the rats became 72 days old or older. Thus 7 of the original 13 vegetarians were transferred to omnivorous diets before they became 200 days old and, because of the deterioration of some of the remaining 6, all of them were placed on omnivorous diets by the time they became 345 days old. Nevertheless 10 rats (5 males and 5 females) started on omnivorous diets were placed on diet 6 after they became 72 to 200 days old and were kept on this diet until they died. Seventy of the rats in this study were fed the diets ad libitum and 45 were fasted 1 day in 3 after they became 100 to 200 days old.

In addition to observations on the growth of rats made during the 2 longevity studies, short-term growth studies were made on other Wistar rats and on Sprague-Dawley rats. In these studies, diet 1 and the Sherman and Campbell diet (containing 1 part whole milk powder and 2 parts whole wheat flour with 1.33% added NaCl) plus lettuce ad libitum daily were used

as the omnivorous diets and diets 6, 6C and 6CC (table 1) were used as the vegetarian diets.

RESULTS

First longevity study

Figure 1 (first study) shows that the rats fed our basic omnivorous diet (diet 1) ad libitum attained weights about 30% (females) to 35% (males) greater than the average

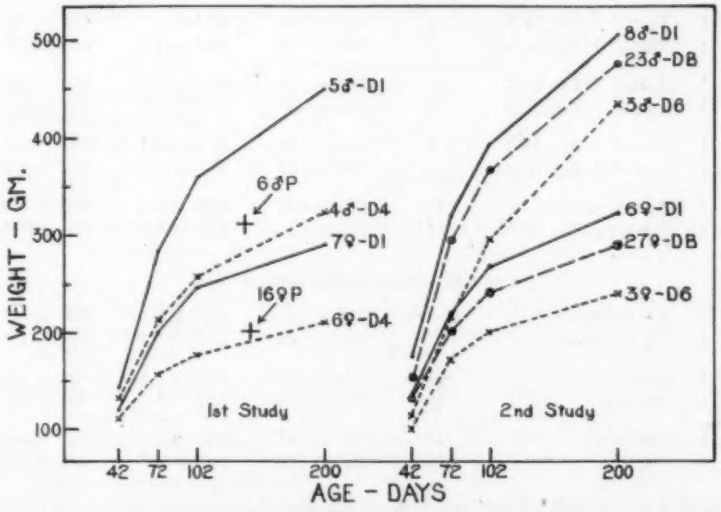

Fig. 1 Composite growth curves of rats fed omnivorous diets (D1 and DB) and vegetarian diets (D4 and D6) in 2 longevity studies. D1 = diet 1, DB = diet 1 with 10% added bulk-formers, D4 = diet 4 and D6 = diet 6. The crosses between the growth curves of the rats in the first study indicate the average weights and ages of the parent rats when they were received from The Wistar Institute.

weights of the parent rats when they were received from The Wistar Institute. On the other hand, the rats fed the vegetarian diet (diet 4), although fed this diet exclusively only after they became 42 days old, weighed less than the parent rats at similar ages. The average maximum weights attained

TABLE 2

Maximum weights, femoral lengths and ages at death of rats fed an omnivorous diet (diet 1) and rats fed vegetarian diets (diets 4 and 6), ad libitum or with intermittent fasting, during 2 longevity studies.

DIET	REGIMEN	NO. OF RATS	MAXIMUM WEIGHT [1] gm	FEMORAL LENGTH [1] mm	AGE AT DEATH [1] Days
		Males — First longevity study			
1	Fed ad libitum	5	591 ± 212	39.0 ± 1.5	633 ± 123
4	Fed ad libitum	5	354 ± 59	38.4 ± 0.5	595 ± 146
1	Fasted 1 day in 4	5	493 ± 107	39.0 ± 1.2	660 ± 91
4	Fasted 1 day in 4	5	350 ± 60	38.4 ± 1.1	685 ± 238
1	Fasted 1 day in 3	4	379 ± 96	38.4 ± 1.2	649 ± 225
4	Fasted 1 day in 3	6	318 ± 62	37.2 ± 1.7	637 ± 214
1	Fasted 1 day in 2	5	348 ± 31	37.0 ± 0.7	691 ± 112
4	Fasted 1 day in 2	5	250 ± 57	34.9 ± 1.1	607 ± 199
1	All regimens	19	453 ± 120	38.4 ± 1.1	659 ± 128
4	All regimens	21	323 ± 56	37.2 ± 1.1	631 ± 187
		Littermate males only			
1	All regimens	7	363 ± 85	37.7 ± 0.8	629 ± 127
4	All regimens	7	264 ± 27	35.7 ± 1.2	572 ± 170
1	Controls [2]	12	474 ± 179	38.5 ± 1.1	600 ± 105
1	Fasted [2]	12	394 ± 105	38.0 ± 1.5	680 ± 154
4	Controls [2]	13	348 ± 67	38.0 ± 1.4	628 ± 180
4	Fasted [2]	13	296 ± 66	36.5 ± 1.8	674 ± 191
		Females — First longevity study			
1	Fed ad libitum	7	456 ± 187	34.7 ± 0.2	659 ± 125
4	Fed ad libitum	6	236 ± 33	33.9 ± 0.8	649 ± 232
1	Fasted 1 day in 4	7	343 ± 38	35.1 ± 0.8	685 ± 101
4	Fasted 1 day in 4	7	215 ± 50	34.3 ± 0.8	545 ± 236
1	Fasted 1 day in 3	7	351 ± 60	34.9 ± 1.1	834 ± 138
4	Fasted 1 day in 3	7	239 ± 43	34.7 ± 0.6	762 ± 190
1	Fasted 1 day in 2	5	280 ± 56	34.5 ± 0.8	754 ± 103
4	Fasted 1 day in 2	5	200 ± 21	32.6 ± 0.7	643 ± 214
1	All regimens	26	359 ± 100	34.8 ± 0.8	733 ± 112
4	All regimens	25	223 ± 34	33.9 ± 0.7	650 ± 204

TABLE 2 *(continued)*

DIET	REGIMEN	NO. OF RATS	MAXIMUM WEIGHT [1] gm	FEMORAL LENGTH [1] mm	AGE AT DEATH [1] days
		Littermate females only			
1	All regimens	18	333 ± 58	34.7 ± 0.8	723 ± 154
4	All regimens	18	214 ± 33	33.8 ± 1.0	630 ± 214
1	Controls [2]	18	427 ± 165	34.8 ± 0.4	702 ± 126
1	Fasted [2]	18	335 ± 59	35.0 ± 0.8	753 ± 131
4	Controls [2]	16	226 ± 41	34.2 ± 0.7	619 ± 248
4	Fasted [2]	16	230 ± 44	33.9 ± 1.3	645 ± 152
		Males — Second longevity study			
1	Fed ad libitum	4	639 ± 169	39.3 ± 0.6	588 ± 96
6–1 [3]	Fed ad libitum	1	570	37.8	511
1–6 [4]	Fed ad libitum	1	524	40.9	564
1	Fasted 1 day in 3	4	534 ± 86	38.2 ± 1.1	589 ± 206
6–1 [3]	Fasted 1 day in 3	0			
1–6 [4]	Fasted 1 day in 3	1	458	38.3	470
		Females — Second longevity study			
1	Fed ad libitum	3	551 ± 72	33.2 ± 0.5	701 ± 129
6–1 [3]	Fed ad libitum	1	244	32.1	379
1–6 [4]	Fed ad libitum	1	362	34.3	585
1	Fasted 1 day in 3	3	357 ± 63	34.1 ± 1.5	779 ± 298
6–1 [3]	Fasted 1 day in 3	0			
1–6 [4]	Fasted 1 day in 3	1	330	32.9	835

[1] Mean and standard deviation.

[2] The controls include rats fed ad libitum and rats fasted 1 day in 4 or 3 but all fasted control rats fasted less than their fasted littermates.

[3] Diet 6 fed early in life and diet 1 fed later in life.

[4] Diet 1 fed early in life and diet 6 fed later in life.

on the 2 diets and 4 regimens are presented in table 2. The greatest individual weights attained by rats fed the diets ad libitum were: male on diet 1, 870 gm, male on diet 4, 450 gm, female (with mammary tumor) on diet 1, 855 gm, and female, (with gastric and uterine tumors) on diet 4, 294 gm. Figure 2 and table 2 show that the weights attained by the intermittently fasted rats were inversely proportional to the

amount of fasting. The sizes attained, as indicated by femoral length at death (table 2), were more uniform than the weights. The ages attained (fig. 2 and table 2) were, on the average, greater in rats fed diet 1 than in rats fed diet 4 and greater in fasted rats than in rats fed ad libitum. Mammary tumors developed in 8 (31%) of the females on diet 1 but only in 2 (8%) of the females on diet 4. Moreover, the mammary tumors developing in females fed diet 1 were much larger than those in females fed diet 4 — the largest tumor in a female fed diet 1 (462 gm) weighed much more than the heaviest female (with tumors) fed diet 4 (294 gm).

Second longevity study

Figure 1 (second study) shows that the feeding of the different experimental diets earlier in life than in the first study produced considerable divergences in the weights already at the age of 42 days. The omnivorous rats weighed more and the vegetarians less at 42 days than the rats in the corresponding groups in the first study but the vegetarians as well as the omnivores in the second study attained greater weights, by the time they became 200 days old, than the corresponding rats in the first study. In fact, the males fed the vegetarian self-selection diet (diet 6) ad libitum gained more weight between the ages of 72 days and 200 days than the rats in this study fed diet 1 or diet 1 plus bulk-formers ad libitum (fig. 1). However, the rats started on diet 6 that were fasted 1 day in 3 did not seem to fare as well as rats fed diet 4 and fasted 1 day in 3 in the first longevity study. The rats fed diet 6 ad libitum also began to lag considerably in growth, in comparison with the rats on the omnivorous diets, after they became 200 days old. Early deaths among the intermittently fasted vegetarians seemed likely. Hence, all of the rats started on diet 6 were transferred to omnivorous diets. Rapid improvement in the deteriorated rats followed. Rats that were started on the omnivorous diets and transferred to diet 6 when they were between 72 and 200 days old tolerated fasting

1 day in 3 better. Table 2 includes data on rats in the second longevity study that were fed diet 1 throughout life and rats that were fed diet 6 before or after diet 1. The data indicate that the rats fed diet 6 early in life or late in life did not grow as well or live as long as the rats fed diet 1 throughout life. The maximum weights attained by individual rats in the second longevity study were: male on diet 1, 890 gm, male on diet 6 (early in life), 570 gm, female (with mammary tumor) on diet 1, 574 gm, and female (with ovarian tumor) on diet 6

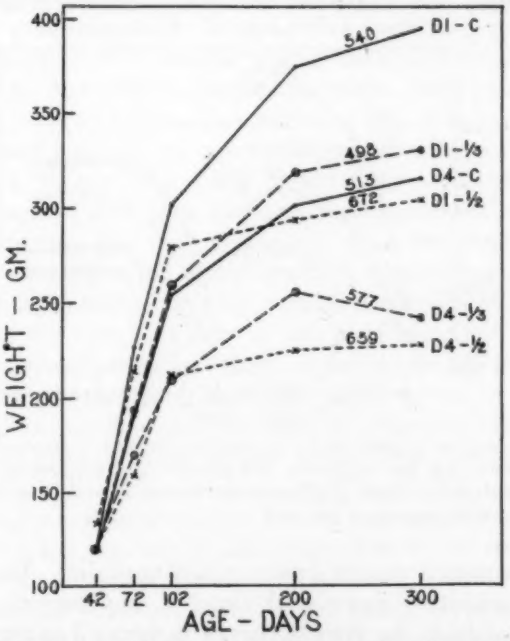

Fig. 2 Composite growth curves of 2 littermate trios of male rats fed the basic omnivorous diet (D1) and 2 trios fed the vegetarian diet (D4) with 1 of the littermates in each trio fed the diet ad libitum (control or C) 1 fasted 1 day in 3 (1/3) and the third fasted 1 day in 2 (1/2). The trios on D1, however, were not littermates of the trios on D4. The figures on the curves indicate the average age attained by the 2 rats on each regimen. The decrease between the ages of 200 and 300 days in the average weight of the 2 rats on D4 and fasting 1 day in 3 was due to the early deterioration of 1 of these rats.

(late in life), 362 gm. Four of the 6 females fed diet 1 throughout life developed mammary tumors; none of the 3 females fed diet 6 early or late in life and diet 1 late or early in life developed such tumors.

Short-term growth studies

Alternate litters raised from the same (Wistar or Sprague-Dawley) rats on either diet 1 or the Sherman and Campbell diet plus lettuce daily showed that the weights attained on the Sherman and Campbell diet were uniformly lower than

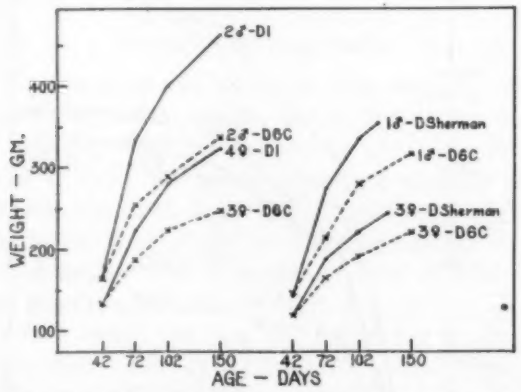

Fig. 3 Composite growth curves of rats fed the basic omnivorous diet (D1) with littermates fed the vegetarian self-selection diet supplemented by celery cabbage as well as by lettuce (D6C) and rats fed the Sherman and Campbell diet (D Sherman) with littermates fed D6C.

weights attained on our basic omnivorous diet. The relative growth-promoting powers of these 2 omnivorous diets can be inferred from the growth curves in figure 3 as the effect on littermate rats was similar to the depicted effects on rats belonging to different litters. A comparison of the growth curves in figure 3 with the growth curves of the rats represented in figure 1 also shows that the inclusion of celery cabbage in the vegetarian diet did not significantly improve growth in comparison with growth on omnivorous diets.

DISCUSSION

The great weights attained by some of the rats on our basic omnivorous diet (diet 1) seem explainable by the nature of the diet. This diet was designed to supply meat from young animals (calves) as the chief source of protein and to include enough protein to meet the assumed requirements of intermittently fasted rats. The dried meat ("whole veal"), as originally prepared for us by Swift and Company, contained about 50% protein and 40% fat. A diet containing about 35% protein seemed to be needed and the fat in the "whole veal" made the diet practically a paste diet including about 28% fat. In short, diet 1 became a diet rich in animal protein and fat like the growth-promoting paste diet which is included in the Anderson and Smith ('32) diet. Moreover, although our aim was to use mainly natural foods, we used corn starch rather than whole wheat flour as the source of carbohydrate in diet 1 to avoid introducing more vegetable protein into this diet than necessary. As a consequence, diet 1 became a concentrated diet containing a minimum of roughage. Diet 1 undoubtedly also appealed considerably to the appetite of the rats and growth was approximately as rapid as the growth observed by others on the Anderson and Smith diet (Dunn, Murphy and Rockland, '47).

The vegetarian diet used in our first longevity study (diet 4) was designed to make whole wheat flour the chief component. The other components served mainly to increase the protein content of the diet. Diet 4 contained about 30% protein and 8% fat but because of the difference in fat content between diet 4 and diet 1, diet 4 supplied more protein than diet 1 on an isocaloric basis. Diet 4, incidentally, contained much more roughage than diet 1 and our bulky omnivorous diets were designed to determine the effect of the bulkiness of vegetarian diets on growth and longevity. Figure 1 (second study) indicates that less than half of the lower weight of vegetarians is accounted for by the bulkiness or non-nutritive fraction of a vegetarian diet like diet 6. The relatively poor growth of rats on a vegetarian diet is therefore mainly due to

the unsatisfactory nature of the nutritive components of the diet.

The higher general weight levels attained by rats in the second life span study were evidently mainly due to the use of only second and later litters and limiting the size of the litters. Seasonal differences in the growth rates of rats on diet 1 and diet 6 were found to explain the relatively more rapid growth of the vegetarian males in the second longevity study between the ages of 72 days and 200 days. That is, the vegetarians fared best in summer while the rats on diet 1 grew most rapidly in cooler seasons. Our rat quarters were not air-conditioned and the period when the vegetarians were between 72 and 200 days old included the summer period.

The great variations in the maximum weights of the rats fed diet 1 ad libitum (indicated by the standard deviations in table 2) are only partly explainable. In the first longevity study, the variations in maximum weights were largely due to variations in initial weights. The ages attained before growth ceased also varied more in the first study than in the second. The failure of the males in the second longevity study to attain more uniform maximum weights suggested that a rich diet like diet 1 fed ad libitum may have some deteriorating effect on successive generations but a further study of this possibility will be necessary before definite conclusions can be drawn. The maximum weights of the females on diet 1 varied mainly because of differences in the occurrence of and size attained by mammary tumors. It became evident in the second longevity study that the size attained by the mammary tumors depended partly on their location. Mammary tumors which interfered with eating, defecating or urinating were usually chewed off by the rats.

The great variations in the life span of the rats, particularly of the vegetarians fed diet 4 (table 2) naturally raised the question whether the life spans of the rats fed diet 1 were significantly greater than the life spans of the rats fed diet 4. The data on littermate males alone and littermate females alone did not show a significant difference but the combined

littermate male and female data showed that the omnivorous rats lived significantly longer ($P < 5\%$). However, the greater variations in the life spans of the vegetarian rats furnish a basis for the widely held impression that vegetarianism prolongs life. That is, although the vegetarian rats, on the average, died sooner than the omnivorous rats, 4 vegetarians became over 1000 days old while only 1 omnivorous rat became over 1000 days old. Moreover, the ageing vegetarians looked younger than the ageing omnivorous rats — the vegetarians did not develop skin lesions and alopecia like most of the old omnivorous rats.

The effect of intermittent fasting on the life spans also indicated that the omnivorous diet was superior to the vegetarian diet. The combined data on 12 pairs of littermate males and 18 pairs of littermate females fed diet 1 (table 2) showed that the fasted littermates lived significantly longer than their controls (average, 62.8 days longer — $P < 2\%$). In contrast to this, 13 pairs of littermate males together with 16 pairs of littermate females fed diet 4 failed to show that the fasted littermates lived significantly longer (average, only 34.6 days longer — $P > 50\%$). In our preceding report ('46), the view was expressed that fasting 1 day in 3 was the optimum amount of fasting for rats. Fasting 1 day in 4 did not seem to be of significant value. Fasting 1 day in 2 on the other hand seemed to be too much fasting. Figure 2, for example, shows that the growth of both omnivorous and vegetarian rats fasted 1 day in 2 tended to be stunted after they became about 100 days old and an amount of fasting or food restriction which stunts growth must be regarded as too much fasting or too much food restriction even if the life span (or mere existence) is thus prolonged. Fasting 1 day in 3 did not significantly stunt growth (size as indicated by femoral length) although it limited weight and prolonged life. Twelve of our rats fed diet 1 ad libitum or that were fasted only 1 day in 4 had littermates fed the same diet and fasted 1 day in 3. The littermates fasted 1 day in 3 lived significantly longer (average, 99 days longer —

$P<2\%$). Similar data on 10 pairs of littermates fed diet 4 did not show that the littermates fasted 1 day in 3 lived significantly longer. Actually the vegetarians fasted 1 day in 3 lived, on the average, 105 days longer than their littermate controls and this suggests that fasting 1 day in 3 was also the optimum amount of fasting for the vegetarians but the variations in the life spans of the vegetarians were so great that a much larger number of rats would have been necessary to show that even an average of 105 days longer life represented a significant increase in the life span ($P>50\%$).

The data secured during our second longevity study were too meager to be treated statistically but it can be seen (table 2) that the results were similar to those obtained during our first longevity study. The results can therefore be regarded as additional evidence that a purely vegetarian diet tends to shorten life and that fasting 1 day in 3 tends to prolong life. This will become clearer when the results of feeding diet 1 with added bulk are reported.

A possible criticism of our longevity studies is that the vegetarian diets were not supplemented by a suitable fresh leafy vegetable. Wu and Wu ('28) found that the kinds and proportions of grains and legumes in vegetarian diets had no significant influence on the adequacy of such diets but that the type of fresh leafy vegetable supplement used was of considerable importance. Lettuce was found to be a poor leafy vegetable supplement to a vegetarian diet. Wu and his associates used colza or Chinese "small cabbage" in their extensive studies. Colza and Chinese "small cabbage" were not obtainable in local markets. We supplied the rats with various other greens besides lettuce from time to time but the rats preferred lettuce. Hence, we fed mainly lettuce as the supplement to all diets. However, after Adolph ('44) stressed the value of celery cabbage as a supplement to a diet of grains and legumes we tried this in short-term growth studies on Wistar and Sprague-Dawley rats. The results have not convinced us that the celery cabbage obtainable in local markets improves the growth of rats or would be likely to signifi-

cantly influence the life span. The rats preferred lettuce to celery cabbage when both were provided and also did not eat much celery cabbage when no lettuce was provided.

The observed results on the rats confirm the personal experiences of 1 of us (H) that a purely vegetarian diet (chiefly a fruit and nut diet) was unsatisfactory, that fasting was more easily borne and apparently of more benefit when an omnivorous diet was used and that fasting 1 day in 2 was too much fasting regardless of diet.

SUMMARY

The growth of rats fed omnivorous and vegetarian diets was observed during 2 longevity studies and during additional short-term studies.

Two of 9 male Wistar rats attained weights of 870 and 890 gm, respectively, on an omnivorous diet including about 35% protein (chiefly meat protein) and 28% fat.

Rats fed purely vegetarian diets were stunted in growth.

Rats fed an omnivorous diet lived significantly longer than rats fed a vegetarian diet. Moreover, the life span was more uniformly prolonged by the intermittent fasting of rats fed an omnivorous diet than in rats fed a vegetarian diet.

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EXPERIMENTS WITH DELAYED SUPPLEMENTATION OF INCOMPLETE AMINO ACID MIXTURES[1]

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FOUR FIGURES

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The optimal composition of amino acid mixtures for therapeutic uses is still under investigation. The logical way to determine the optimal composition appears to be to calculate statistically from the analysis of a so-called "normal" diet the quality and relative quantity of the amino acids present in the average daily protein intake (Block, '43; Mitchell and Block, '46). An amino acid mixture which duplicates the composition of such an average daily protein supply could *a priori* be regarded as satisfactory. Against such a conclusion, however, serious objections must be raised.

The dietary proteins are digested slowly, and therefore the absorption of amino acids takes place gradually, according to the rate of enzymatic liberation. On the other hand, after intravenous injection of amino acid mixtures, the organism is suddenly flooded with these compounds; on feeding of amino acid mixtures differences in rates of intestinal absorption and the rate of renal clearance of amino acids have to be considered (Chase and Lewis, '34; Goettsch et al., '44; Eaton and

[1] The data of this paper were taken from a thesis submitted by the author to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] With the technical assistance of Leslie E. Geiger.

Dotty, '41). It is possible, therefore, that even in cases where amino acid mixtures of optimal composition are fed, they may never be present in such relative optimal concentrations simultaneously in the tissues as required for protein synthesis.

The fate of such amino acid mixtures, which by absolute or relative lack of certain constituents were either not at all or only partially utilized for protein synthesis, is, therefore, worthy of investigation. Three possibilities were considered: (1) that the amino acids can be stored in the body as such, representing thereby a pool from which protein synthesis can proceed as soon as the lacking building stones are supplemented; (2) that they are stored in the form of osmotically less active peptides; and (3) that they are irreversibly further metabolized, and are therefore not available for protein synthesis.

The first 2 possibilities were investigated in experiments described elsewhere (Geiger, '47). In those experiments it was not possible to detect any transitory storage of incomplete amino acid mixtures in the form of either amino acids or peptides. It was decided, therefore, to investigate by an indirect method whether administered amino acid mixtures, which by lack of 1 or more essential factors cannot be utilized for protein synthesis, are stored in some other way, so that after supplemental administration of the lacking amino acid protein synthesis can proceed. More specifically, it was planned to feed to young rats a diet lacking 1 or more essential amino acids during 12 hours, and then, in the next 12 hours to provide the missing amino acids by feeding a mixture containing besides carbohydrates, fats, vitamins, and salts, as the source of N *only* the amino acids which were missed from the preceding feeding.

It was assumed that if the incomplete mixture is stored in the body, protein synthesis and body growth will occur after the missing building stones have been provided. If, however, before feeding the supplement, the incomplete amino acid mixture was already in some way metabolized (deamination, gluconeogenesis, oxidation), then at the time of addition of

the missing building stones certain of the components of the incomplete diet would not now be available and, therefore, the animals would not show any growth.

METHODS

The amino acid mixture used in the experiments with tryptophane was prepared by hydrolysis of mackerel protein. This protein, as was shown by the author in collaboration with Deuel and others ('46), has a higher biological value than casein. The protein was hydrolyzed with sulfuric acid at 15-pound pressure for 8 hours. After careful removal of the sulphuric acid by barium hydroxide, the solution was concentrated under reduced pressure to a total solid content of 15%. Since the tryptophane originally present in the protein was destroyed during the acid hydrolysis, the solution prepared by this procedure was an incomplete amino acid mixture. This solution was then spray dried. When fed to rats of 40–50 gm body weight, at a level of 3% N as the sole source of protein, it did not promote growth, which would presumably indicate the absence of an essential amino acid. This incomplete mixture, when supplemented with 1% *l*(—) tryptophane, had a normal growth-promoting activity evidenced by an average daily growth of 4–5 gm.

The composition of the various diets used in these experiments is given in table 1.

For the experiments the pure bred Sprague-Dawley rats were placed in individual cages provided with wire mesh bottoms. The main difficulty encountered was with respect to determination of the daily food intake, due to the habit of some rats of spilling the feed. The following satisfactory arrangement was finally devised: Ointment jars of 40 mm diameter and 40 mm height with a metal screw cap having a circular opening of 25 mm diameter, were employed as food containers. The food was placed in the jar and covered with a piece of wire screen (1 × 1 cm mesh). The jar was then placed in a glass dish of 110 mm diameter and 60 mm height.

The whole system was always weighed together, so that small amounts of feed spilled into the outer jar were not lost. Besides these precautions, rats which did not have the habit of spilling their food were selected for these experiments.

TABLE 1

Composition of diets.

COMPONENT	DIET NUMBER											
	1	2	3	4	5	6	7	8	9	10	11	12
Basal diet [1]	78.4	70	94	18.3	70	95	94	68	65	79	96	84.5
Acid hydrolyzed fish protein	19.5	30	..	...	..	..	..	..	..	..	..	...
Casein, oxidized	...	..	..	...	..	..	..	..	..	19	..	14
Zein	...	..	..	17.0	30	..	..	30	30	..	..	...
l(—)Tryptophane	2.1	..	6	0.5	..	5	..	2	..	1	..	0.5
l(+)Lysine	...	..	..	1.2	..	..	6	..	5	..	..	...
l(—)Cystine	...	..	..	...	..	..	..	..	..	1	..	0.5
dl-Methionine	...	..	..	...	..	..	..	..	..	..	4	0.5

[1] With the following composition: corn starch, 3050 gm (78.3%); rice bran concentrate, 400 gm (10.26%); cottonseed oil, 200 gm (5.13%); USP salt mixture, 200 gm (5.13%); fish oil (1 gm contains 2,000 I.U. vitamin A and 400 I.U. vitamin D) 50 gm (1.27%); riboflavin, 75 mg; Ca pantothenate, 150 mg; choline chloride, 2.5 gm.

EXPERIMENTS

Experiments with tryptophane

In the first group of experiments, tryptophane-free diets were fed, followed by a diet supplemented with the missing tryptophane. Rats of group I received the complete diet (no. 1) containing tryptophane and all other amino acids. The rats of group II had 2 jars in the cage, 1 containing diet 2 (lacking tryptophane) and the other containing diet 3 (containing the basal mixture plus tryptophane but lacking the acid hydrolyzed fish protein). Group III received for 12 hours diet 2 and in the next 12 hours diet 3. In order to rule out the effect of daylight on food intake, since rats are nocturnal animals, 1 of the rats received diet 2 during the day and diet 3

during the night and the other had diet 3 available during the daytime and diet 2 during the night.

The rats of group I, which received a complete diet (no. 1), served as controls. In group II, the rats had the chance to choose their food from the 2 jars; and on the basis of the experiments of Richter ('42–'43) concerning the behavior of rats in the self-selection of food, it was expected that the rats of this group would take from both jars such quantities that the food consumed *in toto* would support optimal growth. The rats of group III received the tryptophane-free amino acid mixture and the missing tryptophane at a 12-hour interval.

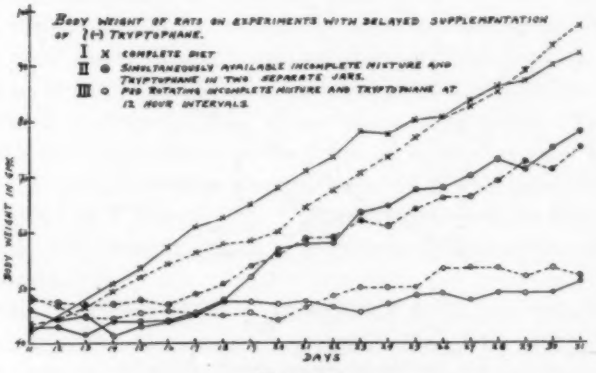
BODY WEIGHT OF RATS ON EXPERIMENTS WITH DELAYED SUPPLEMENTATION OF (+) TRYPTOPHANE.
I × COMPLETE DIET
II ● SIMULTANEOUSLY AVAILABLE INCOMPLETE MIXTURE AND TRYPTOPHANE IN TWO SEPARATE JARS.
III ○ FED ROTATING INCOMPLETE MIXTURE AND TRYPTOPHANE AT 12 HOUR INTERVALS.
BODY WEIGHT IN GMS.
DAYS

Figure 1

It was observed, however, that the rats did not consume the food immediately after it was put into the cages and therefore, the interval between intestinal absorption of the incomplete amino acid mixture and of the supplemented tryptophane varied considerably.

Figures 1 and 2 are self-explanatory. It is evident that the rats receiving the complete diet grew satisfactorily. The rats in group II which had the opportunity of supplementing diet 2 with diet 3, having both diets simultaneously present in different jars in the cage, did not grow for the first 6–7 days. In both experiments growth starts after this preliminary ad-

justment period. A highly probable explanation for this observation is that after 5–6 days the rats learn to eat food rotating from the 2 jars at shorter intervals, so that an effective supplementation may be achieved. This possibility should be investigated in further experiments in which the time when the rats eat from the individual jars would be registered automatically. Finally, it is seen that there was no growth in group III in spite of the fact that the rats consumed considerable quantities of both diets 2 and 3.

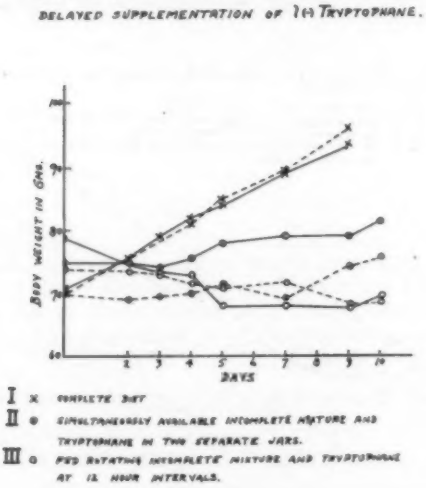
BODY WEIGHT OF RATS ON EXPERIMENTS WITH DELAYED SUPPLEMENTATION OF 1% TRYPTOPHANE.
BODY WEIGHT IN GMS.
DAYS
I × COMPLETE DIET
II ○ SIMULTANEOUSLY AVAILABLE INCOMPLETE MIXTURE AND TRYPTOPHANE IN TWO SEPARATE JARS.
III ○ FED ROTATING INCOMPLETE MIXTURE AND TRYPTOPHANE AT 12 HOUR INTERVALS.

Figure 2

These experiments are interesting with respect to the ability of rats to select foods properly. Values of the ratio $\frac{\text{ingested diet 2}}{\text{ingested diet 3}}$ were 2.3, 2.38, 1.78, and 2.5, which indicate that the rats selected an average of 1.0 gm of the tryptophane containing supplement for 2.24 gm of the tryptophane-deficient food. Figure 2 gives the results of a similar experiment of shorter duration.

The results of these experiments with tryptophane show that a delayed supplementation of tryptophane is ineffective;

this amino acid does not promote growth when fed several hours after ingestion of a tryptophane-deficient diet. These results are in excellent agreement with the observation of Elman ('39) who found that when the injection of an amino acid solution which is deficient in tryptophane is followed after a 6-hour interval by the intravenous injection of tryptophane, positive N-balance cannot be achieved. Several authors have assumed that the utilization of parenterally administered tryptophane is different from that administered *per os* (for literature see Alcock, '36); therefore, the results obtained in our experiments with oral administration seem to be a significant confirmation of Elman's finding.

Somewhat similar experiments were published in 1929 by Berg and Rose. These authors showed that feeding large doses of tryptophane at long intervals (24-hour) is less effective than feeding smaller doses more frequently. Feeding half the daily allowance in the form of tablets at intervals of 12 hours induced better growth than when the total was administered at 1 time daily. The authors conclude that "apparently amino acids ingested in excess of anabolic needs are not retained."

Before drawing further conclusions from our results, we had to consider that according to the literature tryptophane is very rapidly eliminated from the body. Buck and Berg ('45) found that after oral administration of 0.35 to 1.0 gm tryptophane the tryptophane concentration in the blood increases rapidly, reaches its maximum after 2 hours and is again normal 5 hours after administration. The authors report also, that 1 hour after administration, only one-third of the fed tryptophane could be detected in the carcass. It was therefore assumed that tryptophane is very quickly destroyed either by deamination or by kynurenic acid formation.

Schweigert, Sauberlich, Elvehjem and Baumann ('46) also reported that after intraperitoneal administration of tryptophane, its concentration in the blood increases rapidly but returns to normal again in 2 hours.

In view of these data, it seemed possible that the results obtained in the present experiments with tryptophane were the result of the rapid destruction of this amino acid and may not represent a generally valid answer to the question whether incomplete amino acid mixtures are stored in the body. Therefore, it was decided to investigate whether the results observed in the experiments with tryptophane can be reproduced with other essential amino acids.

Experiments with lysine

A lysine-free protein was readily prepared according to the procedure of Dunn and Lewis ('19). Casein was treated according to the method of these authors and fed as the sole

TABLE 2

Experiments with casein treated according to Dunn and Lewis ('19).

COMPOSITION OF DIET	RAT NO.	BODY WEIGHT (GM) ON DAY OF FEEDING					
		Before	1	2	3	4	6
"Basal diet" 80 gm and	1	42	40	35	31	29	27
treated casein 20 gm	2	37	35	31	27	23	died
	3	106	104	86	72	65	60
"Basal diet" 80 gm,	4	44	40	35	30	27	25
treated casein 20 gm,	5	40	40	34	28	died	
and lysine 1.5 gm	6	120	116	102	88	77	63
"Basal diet" 70 gm,							
treated casein 10 gm,	7	115	108	95	82	77	75
and casein 20 gm	8	106	100	85	67	60	52
"Basal diet" 70 gm,							
casein 20 gm, and							
celluflour 10 gm	9	115	121	126	130	137	145

protein source to rats. The data in table 2 show that rats fed such a diet lose weight very fast (see experiments 1, 2 and 3). It was assumed that lack of lysine was responsible for the weight loss and, therefore, the next group of rats was fed the same diet with the addition of 1.5 gm lysine to each 100 gm of the diet. These rats (experiments 4, 5 and 6) lost

weight in spite of the lysine supplementation, and this weight loss was more rapid than that observed by us earlier on rats fed diets entirely devoid of protein. It was assumed, therefore, that during the nitrite treatment, toxic substances were produced and that the presence of such substances rather than the destruction of lysine was responsible for the weight loss. This assumption was verified by experiments in which 10 gm of the treated casein were added to a regular diet containing 20 gm casein and 80 gm "basal diet." From experiments 7, 8 and 9 (table 2) it is evident that the control animal (no. 9) fed 20% casein and 80% "basal diet" gained as was to be expected, but the other animals (nos. 7 and 8) responded to the addition of nitrite-treated casein with loss of weight. The presence of toxic factors in casein treated according to Dunn and Lewis was assumed also by Hogan et al. ('41).

Another way to investigate the effect of delayed supplementation of lysine is to use zein as the source of lysine-free protein (Osborne-Mendel, '15; Rose and Rice, '39). This protein has to be supplemented with both tryptophane and lysine in order to secure growth in young rats and maintenance of weight in adult animals (Neuberger and Webster, '45; Gillespie, Neuberger and Webster, '45). The growth-promoting power of zein can be improved by adding to the diet these amino acids but even so, a normal rate of growth cannot be obtained. The reason for such failure has not yet been determined (Harris et al., '43; Borchers et al., '42; Kornberg, '46).

For the lysine-experiments (fig. 3), the rats of group I representing the control group, received diet 4 which contained both zein and the tryptophane and lysine supplements. The rats of this group grew sub-optimally as observed by Harris et al. ('43), and by Borchers et al. ('42).

Rats of group II had 3 jars in the cage simultaneously, 1 with diet 5 containing zein; 1 with diet 6 containing tryptophane, and the third with diet 7 containing lysine. It will be noticed that the rats of this group did not grow; in fact, they

lost weight. The animals did not show any sign of efficient self-selection of food (Scott and Quint, '46). Both preferred diet 7 containing lysine from which they ate relatively large quantities, but the amounts of diets 5 and 6 consumed were too small to permit growth.

The animals in group III were fed diet 8 containing zein and tryptophane for 12 hours, and diet 7 containing lysine for the next 12 hours.

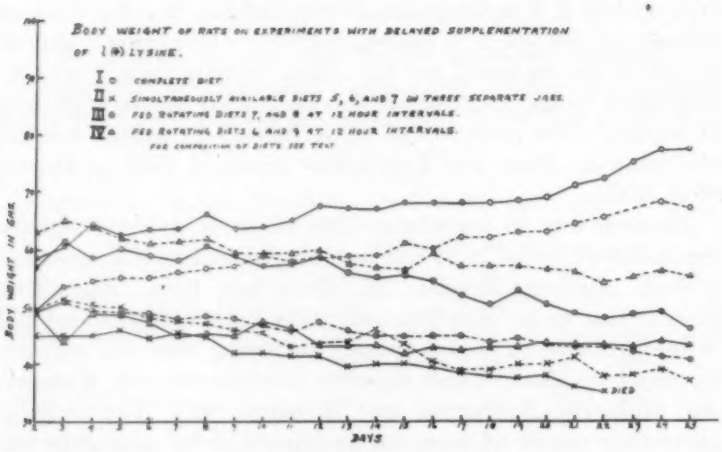
BODY WEIGHT OF RATS ON EXPERIMENTS WITH DELAYED SUPPLEMENTATION OF L (+) LYSINE.
I ○ COMPLETE DIET
II × SIMULTANEOUSLY AVAILABLE DIETS 5, 6, AND 7 IN THREE SEPARATE JARS.
III ○ FED ROTATING DIETS 7 AND 8 AT 12 HOUR INTERVALS.
IV △ FED ROTATING DIETS 6 AND 9 AT 12 HOUR INTERVALS.
FOR COMPOSITION OF DIETS SEE TEXT
BODY WEIGHT IN GMS.
× DIED
DAYS

Figure 3

The rats of this group again conspicuously preferred diet 7 containing lysine but did not show any growth.

Finally, in group IV the animals were fed diet 9 containing zein and lysine for 12 hours, and diet 6 containing tryptophane, for the next 12 hours. In this group, sufficient quantities of both diets were consumed during 24 hours to produce growth if the food were properly utilized, but due to the delayed supplementation, the animals actually lost weight.

These experiments prove that the simultaneous presence of all the amino acids is essential not only in the case of tryptophane, but also with respect to lysine.

Experiments with methionine

A methionine-free casein was prepared according to Toennies ('42). The formic acid hydrogen-peroxide treatment used in this process destroys tryptophane as well as methionine. Therefore, it was necessary to supplement with tryptophane in order to carry out the experiment.

Three groups of rats were used. Control group I received the completed diet 12, and group II received simultaneously

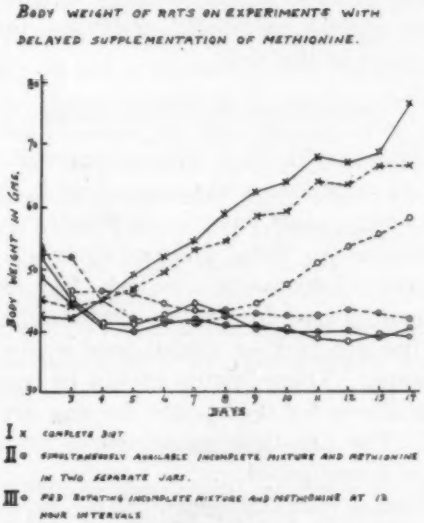
BODY WEIGHT OF RATS ON EXPERIMENTS WITH DELAYED SUPPLEMENTATION OF METHIONINE.
BODY WEIGHT IN GMS.
DAYS
I x COMPLETE DIET
II o SIMULTANEOUSLY AVAILABLE INCOMPLETE MIXTURE AND METHIONINE IN TWO SEPARATE JARS.
III o FED ROTATING INCOMPLETE MIXTURE AND METHIONINE AT 12 HOUR INTERVALS

Figure 4

in 1 jar the methionine-free diet 10 and in the other jar diet 11 containing the methionine supplement. Group III received at 12-hour intervals diet 10 and diet 11 in rotation.

In this experiment, after the eighth day paired feeding for the rats of groups I and II was employed. It was determined how much food was consumed of diets 10 and 11 each by the rats of group II; corresponding quantities were then mixed and fed to the rats of group I next day.

From figure 4 it is evident that the fully supplemented oxidized casein (diet 12) has a fair growth-promoting property

as has already been found by Bennett and Toennies ('42); the rats of group I were growing. However, the animals in groups II and III showed no growth.

One rat of group III, after losing weight for 10 days, suddenly started to regain its weight for no apparent reason.

These experiments with methionine supplementation prove that not only tryptophane and lysine, but also methionine, have to be present simultaneously with the other amino acids in order to provide favorable conditions for protein synthesis and whatever special metabolic processes these essential amino acids serve in the body.

DISCUSSION AND CONCLUSIONS

The experiments with delayed supplementation of lysine, methionine, and tryptophane are concordant in indicating that the essential amino acids have to be simultaneously present for protein synthesis. These findings show also that incomplete amino acid mixtures which by lack of an essential building stone cannot be used for protein synthesis, are not stored in the body but are further metabolized probably in an irreversible manner. These results should be considered when amino acid mixtures for therapeutic feeding are prepared or administered. The practical consequences of these findings will be further investigated.

This observation, that supplementing incomplete amino acid mixtures with the missing amino acid after a certain time interval does not promote growth, seems to constitute an important argument against the theory of step-by-step synthesis of protein molecules with intermediate formation of peptides. These results and those published by Berg and Rose ('29) and by Elman ('39), suggest that the formation of protein molecules is a coordinated tissue function and can be accomplished only when all amino acids which take part in the formation are present at the same time.

Two other papers may be cited in support of such a viewpoint (Melnick et al., '46; Russell et al., '46). The first named

authors explain the different biological values of raw and processed soy protein by the observation, "that during digestion *in vivo* the methionine is released earlier from the heat processed soy meal than from the raw soy meal . . . in the case of raw meal, absorption occurs so late in the intestinal transit that this amino acid, as well as the incompletely supplemented amino acids, are not efficiently utilized for the synthesis of body protein."

Another bit of evidence in support of the assumption that for optimal protein synthesis the simultaneous presence of all the building stones is necessary, was supplied by Cox and Mueller ('44). These authors have shown that less essential amino acids are required for maintenance of N-balance, when the non-essential ones are also provided simultaneously. From their experiments it would seem highly probable that the non-essential amino acids, when not provided along with the essential ones, have to be synthesized in the body *ad hoc*. The synthesis of these building stones requires time during which some of the essential amino acids may be excreted or diverted from the protein synthesis by intermediary degradation or transformation.

SUMMARY

The fate of dietary amino acid mixtures which, because of absolute or relative lack of certain essential constituents, would either not be utilized or only partially utilized for protein formation was investigated.

With delayed supplementation of the lacking amino acid, it was shown that the missing tryptophane, methionine or lysine, when fed several hours after feeding of the "incomplete" mixture, does not promote growth. This finding supports the view that the "incomplete" amino acid mixtures are not stored in the body but are irreversibly further metabolized. It shows also that for protein synthesis all the essential components have to be present simultaneously.

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STABILITY OF REDUCED ASCORBIC ACID IN MARES' MILK[1]

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(Received for publication February 18, 1947)

In a previous study in which Holmes, Spelman, Smith and Kuzmeski ('47) compared the protein, mineral, and ascorbic acid content of mares' milk with that of the milk of other species, it was noted that the ascorbic value was relatively high. Since the mares' milk contained 4 or 5 times as much ascorbic acid as Holmes, Jones, Wertz, Esselen and McKey ('44) and Holmes Lindquist, Jones and Wertz ('45) obtained for milk from a herd of 5 common breeds of cows fed forage produced on the same or similar fields as that consumed by the mares, it was assumed that the difference was due to the 2 species of animals under consideration. This observation raised a question regarding factors that may influence the reduced ascorbic acid content of mares' milk, particularly since a sample of mares' milk that had been stored for several days, possessed approximately the same reduced ascorbic acid content as when it was freshly drawn from the mare, a condition quite different from the reported rapid disappearance of reduced ascorbic acid from cows' and human milk. Accordingly, a study was inaugurated to accumulate data regarding the stability of reduced ascorbic acid in mares' milk.

EXPERIMENTAL

The milk was produced by mature Percheron mares at the end of the lactation period. Although the samples were col-

[1] Contribution No. 617 of the Massachusetts Agricultural Experiment Station.

lected during the late fall, pastures provided nearly all of the forage for the mares. However, since the season was unusually warm and sunny, the rainfall ample, and the pastures had been closely grazed, the grass that the mares consumed was young and green. The mares were in the pasture about 14 hours at night, and during the day they were confined in roomy box stalls where they received a supplementary ration of 4 quarts of cracked oats, a quart of whole corn and a small amount of early-cut, mixed hay. The milk samples, which were collected in the early morning, were protected from the light and taken directly to the laboratory, and the initial assay was started within an hour after the sample was obtained from the mare. The milk was stored in commercial flint glass milk bottles in a non-illuminated refrigerator at 10°C. When the milk was initially placed in storage, the bottles were completely filled and closed with the usual paper milk bottle cap. As aliquots were withdrawn for assay, the volume of air in the bottles constantly increased until when only the last aliquot remained the bottles were almost filled with air. This procedure seemed desirable since, in the average household, milk is frequently stored in the refrigerator in partially filled bottles.

When the study was begun, practically no information was available regarding the stability of the reduced ascorbic acid in mares' milk; therefore some of the samples were assayed daily. Other samples were assayed at longer intervals which served 2 purposes, i.e., materially increasing the period of observation and increasing the duration of the contact of air with the milk. The amount of reduced ascorbic acid in the 15 samples of mares' milk was determined by titration with sodium 2,6 dichlorobenzenonindophenol. A 25 ml sample of milk was measured into a 125 ml flask that contained a mixture of 5% phosphoric acid and 10% acetic acid. For clarification, 0.5 gm of bentonite [2] was added. The flask was stoppered and vigorously shaken for 1 minute and the mixture was filtered.

[2] U. S. Pharmacopoeia, 12th revision, November 1, 1942, Mack Printing Co., Easton, Pa.

The clear filtrate was divided into 10 ml aliquots, cooled in the refrigerator and titrated with the standardized dye solution. The bentonite served both to produce a clear filtrate and to greatly decrease the length of time required for filtering, for the proteins precipitated from mares' milk differ in appearance and physical condition from those of cows' milk. A series of duplicate tests gave the same results whether bentonite was used or omitted.

RESULTS AND DISCUSSION

The results of the individual assays of mares' milk for reduced ascorbic acid are reported in table 1. The range in initial values from 86 mg to 161 mg of ascorbic acid per liter makes it possible to compare the rate of disappearance of reduced ascorbic acid from mares' milk of widely different potencies. Samples VI, IX, XI, and XIII observed for a 10-day period, lost 2.6 mg, 2.3 mg, 2.5 mg, and 2.4 mg of reduced ascorbic acid per day, respectively; samples VII, X, XII, and XV, observed for 20 days, lost 2.5 mg, 1.9 mg, 1.5 mg, and 1.2 mg, respectively; samples III and V, observed for 28 days, lost 1.2 mg and 1.4 mg daily; samples I, II, IV, VIII, and XIV, during 33 days, lost 0.7 mg, 1.1 mg, 2.7 mg, 0.5 mg, and 2.1 mg, respectively. The rate of loss of reduced ascorbic acid from sample IV after 16 days' storage was radically different from that of the other samples. Omitting sample IV, the average daily loss of ascorbic acid from mares' milk stored for 10 days was 2.5 mg per liter, for 20 days was 1.8 mg, for 28 days was 1.3 mg and for 33 days was 1.1 mg. However, this correlation between the daily loss and the initial potency may be merely coincidental.

The 2.5 mg per liter daily decrease in reduced ascorbic acid during the first 10 days of storage agrees well with the 2.2 mg-per-liter loss reported by Gunsalus and Hand ('41) for fresh raw cows' milk. However, the decrease of 2.5 mg of ascorbic acid in mares' milk represents about 1.9% daily loss whereas the 2.2 mg decrease in cows' milk represents 14.8% loss. In other words, the loss of ascorbic acid from

TABLE 1

Stability of reduced ascorbic acid in mares' milk. All values are in mg/l.

AGE OF MILK IN DAYS	SAMPLE I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
0	86	94	99	104	111	116	119	121	123	124	131	147	149	157	161
1	..	..	..	...	...	114	120	...	122	123	130	146	148	157	159
2	..	..	..	104	...	112	119	...	118	122	130	145	145	156	158
3	..	..	93	...	...	109	...	...	116	...	127	...	139	...	...
4	81	92	..	...	104	106	...	...	112	...	125	...	139	153	...
5	..	..	..	100	...	...	111	121	105	117	...	140	132	...	154
6	79	84	..	...	...	...	108	...	...	113	...	138	...	...	150
7	..	..	..	93	...	...	102	...	...	...	...	132	...	...	...
8	..	74	..	...	96	...	...	...	...	...	...	...	...	140	...
9	..	..	78	88	...	93	98	...	...	...	107	129	...	...	...
10	..	..	..	...	...	90	...	...	100	...	106	...	125	119	...
12	71	..	..	...	...		...	...		...		...		...	...
13	70	..	..	...	...		...	...		99		...		...	140
14	..	66	..	...	...		...	...		99		...		...	140
15	..	..	..	...	...		82	117		...		117		...	...
16	..	..	..	85	76		...	117		...		...		110	...
20	..	..	73	...	85		68	...		87		117		...	136
28	..	..	66	...	73			109						102	
33	62	57		16				105						88	
Total loss	24	37	33	88	38	26	51	16	23	37	25	30	24	69	25
Av. loss/dn.	0.7	1.1	1.2	2.7	1.4	2.6	2.5	0.5	2.3	1.9	2.5	1.5	2.4	2.1	1.2

cows' milk is over 7 times as rapid as from mares' milk. In a study of 12 samples of commercial cows' milk stored at 1°C. for 6 days, Hand ('43) found a daily loss of 2.1 mg per liter or a loss of over 11%. Kothavalla and Gill ('43) observed a 24% loss from cows' milk stored at room temperature for 11 hours and a 26% loss during 3 days' storage at 5°C. Holmes and Jones ('45) found either only a trace or no detectable reduced ascorbic acid after a 30-minute exposure to sunshine. These observations are in accord with the data accumulated by several other investigators cited by Holmes and Jones ('45) which show that the reduced ascorbic acid content of cow's milk decreases rather rapidly during the first few days after the milk is obtained from the cow. Few data are available regarding the stability of ascorbic acid in human milk. Stateva ('43) reported that from 41% to 93% was lost during 24 hours' storage at room temperature.

While numerous investigators have supplied data regarding the effect of such factors as air, temperature, light, and oxidation upon the rate of disappearance of reduced ascorbic acid from cows' milk, a question naturally arises as to the primary factor which causes the destruction. Hand, Guthrie and Sharp ('38) reported that "lactoflavin is the sole agent in milk responsible for the sensitivity of ascorbic acid to light." In a study of the pigments, vitamins, and enzymes of milk in relation to changes in flavor and nutritive value, Hand and Sharp ('41) found that "riboflavin, the fluorescent green coloring matter in whey, is responsible for the oxidation of vitamin C in light." Thus one might assume that for a given set of conditions the more there is of naturally occurring riboflavin present in milk, the greater is the extent or the more rapid the disappearance of reduced ascorbic acid. This assumption is in a measure supported by the data under discussion, i.e., reduced ascorbic acid disappears more rapidly from cows' than from mares' milk and cows' milk contains more riboflavin than mares' milk. In one instance reduced ascorbic acid disappeared from cows' milk about 7 times as fast as from mares' milk; and Holmes, McKey, Wertz, Lind-

quist and Parkinson ('46) found cows' milk contained about 10 times as much riboflavin as mares' milk produced on the same farm. Rasmussen ('46) stated that cows' milk contains approximately 10 times as much riboflavin as mares' milk, and Rasmussen, Bogart and Maynard ('38) observed that sunlight did not decrease the ascorbic acid content of mares' milk to the extent that it did for cows' milk. However, it is quite evident that the available data are too meager to permit any definite conclusions regarding any relationship between the amount of riboflavin present in milk and the rate of disappearance of ascorbic acid.

SUMMARY

Fifteen samples of mares' milk with initial potencies of from 86 mg to 161 mg of reduced ascorbic acid per liter were stored in the dark at 10°C. They were assayed at daily or longer intervals. Four samples observed for 10 days lost an average of 2.5 mg per liter daily; 4 samples stored 20 days lost 1.8 mg per day; 2 stored 28 days lost 1.3 mg daily; and 3 observed for 33 days lost 1.1 mg per liter per day. These data show that the rate of loss of reduced ascorbic acid from mares' milk is only a fraction of the rate of loss from cows' milk.

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IS COBALT A DIETARY ESSENTIAL FOR THE RABBIT?[1]

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Of the endemic diseases caused by a deficiency of the micronutrient elements, that due to a lack of cobalt is perhaps the most widespread. In this country alone, localities in Michigan, New Hampshire, and Wisconsin have recently been added to the already long list of cobalt deficient areas (Baltzer et al., '41; Keener et al., '44; Geyer et al., '45). A great deal of work has been done in attempting to clarify the role that this element plays in animal physiology — for recent reviews see Marston ('39), Schultze ('40), Underwood ('40), and Maynard ('41) — yet much remains to be learned. While the decreased and depraved appetite, progressive debility, anorexia, and general emaciation are characteristics of cobalt deficiency in sheep and cattle, none of these symptoms is specific.

Attempts to produce a cobalt deficiency in the rabbit and guinea pig in the hope of finding specific symptoms or changes were unsuccessful. The work is reported briefly since it indicates that there is a species difference in the need for cobalt.

EXPERIMENTAL

The basal diet consisted of whole milk[3] and corn grain.[4] The average of several determinations for the cobalt content

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[3] Obtained from the Cornell University herd, milked into Pyrex jars.

[4] Grown in a low-cobalt area, obtained through the courtesy of Dr. G. K. Davis, Florida Agricultural Experiment Station, Gainesville, Florida.

of the milk was 0.0007 μg/ml, while the corn contained 0.0005 μg/gm dry weight. In the proportions eaten by the animals, this made a diet containing 0.0024 p.p.m. on a dry basis. These analyses and those on liver samples were made by the method of Ellis and Thompson ('45).

Preliminary experiments showed that good growth could be obtained with the rabbit fed this diet, but that with the guinea pig it is unsatisfactory. Sprouting the grain to a length of 6 to 8 inches made a noticeable but insufficient improvement. In this preliminary work it became evident that the requirement for cobalt by both of these species was not high.

A second experiment was performed using rabbits. Twenty animals were distributed at random into 4 groups. From birth to 4 weeks of age they were denied access to the dam's feed, but were allowed to suckle at intervals and were given fluid whole milk and whole milk powder. At 4 weeks of age they were placed in individual glass cages. In addition to the basal diet fed ad libitum, 1 group was given orally twice weekly a supplement of iron, copper, manganese, and cobalt at a rate to give, respectively, 2,000, 200, 2,000 and 4 μg per animal per day. Another group was treated in similar fashion except that the cobalt was omitted. The remaining 2 groups were supplemented only with manganese, since it was expected on the basis of the iron and copper content of the diet that a mild nutritional anemia might be produced and that under these conditions a cobalt deficiency might be more easily demonstrated.

The manganese was purified by the extraction of a solution of manganous chloride with o-nitrosocresol in petroleum ether, while the iron was purified by extraction from strong HCl with diethyl ether according to the method of Dodson et al. ('36). Baker's c.p. cupric sulfate and cobaltous sulfate were used as the source of copper and cobalt.

The animals were weighed weekly and their condition noted. Hemoglobin determinations were made periodically by the method of Sanford and Sheard ('30). After 46 weeks on the

diet, the animals were sacrificed and the livers taken for cobalt analysis.

RESULTS AND DISCUSSION

At 4 weeks of age the hemoglobin values were below normal, ranging between 5 and 9.9 gm/100 ml of blood, because until this age the animals had received only milk. The hemoglobin values for the animals receiving the complete mineral supplement, as well as for those of the animals receiving the supplement lacking only cobalt, rose to normal in about 2 weeks after starting the supplement, while the values for the 2 groups receiving a supplement of manganese alone rose more slowly. After 8 weeks, at which time the hemoglobin levels of the latter 2 groups were still subnormal, 1 of these was given a supplement of iron and the other of copper. This was done to determine if possible whether the anemia found was due to the lack of copper or of iron.

The hemoglobin levels rose slightly, the animals of the group receiving iron responding a little more quickly than those receiving copper; however, the difference in response was small and probably of no significance. At 14 weeks of age the iron and copper supplement was given to all groups; from this point on, then, the 15 surviving animals can be considered as falling into 2 groups, 1 group of 5 receiving cobalt and the other of 10 receiving no cobalt. The groups were not divided equally because it was hoped that if cobalt deficiency symptoms should develop, there would be an opportunity to subdivide the larger group and supplement the diet of part of them with cobalt. The hemoglobin values from this point to the end of the experiment were 12.9 for the group receiving cobalt and 12.6 gm/100 ml for those not receiving cobalt. These values are not significantly different and are somewhat below the value of 14.0 gm/100 ml characteristic of our colony on a stock diet.

At 6 months of age, the 4 surviving females were bred. Three had young, none living for more than a few days. Later attempts to obtain viable young led to similar results.

Although growth was fairly good (see table 1) the mortality rate during the experiment was high, there being 10 survivors at the end of the 46 weeks. Various attempts to reduce the mortality rate by supplemental feeding of sodium chloride, thiamine, and cellulose seemed to be of no help. Although growth was slightly more rapid and the mortality rate slightly lower in the cobalt-supplemented group, the difference was not significant.

TABLE 1

Average weight and number of survivors at monthly intervals.

MONTHS ON EXPERIMENT	COBALT SUPPLEMENT		NO COBALT SUPPLEMENT	
	Wt. gm	No.	Wt. gm	No.
0	336	5	343	15
1	618	5	589	14
2	893	5	712	12
3	1260	5	1053	10
4	1495	5	1242	8
5	1560	5	1384	8
6	1649	4	1400	7
7	1719	4	1435	7
8	1835	4	1753	7
9	1866	4	1728	7
10	1944	3	1848	7
10.5	1906	3	1768	7

A record of the feed consumption was kept for a period of 8 months starting from the beginning of the experiment. Four of the animals fed the cobalt supplement and 7 of those not receiving cobalt survived this period. In the former group the average consumption of corn by each rabbit per day was 34 gm, while the corresponding figure for the latter group was 29.5 gm. The corresponding values for milk consumption were 140 gm and 129 gm per animal per day. Thus, the slightly better growth response of the group receiving cobalt corresponds with a slightly greater feed consumption, but here again the difference is not significant. During this period of 8 months, the average daily intake of cobalt of the non-supplemented group was 0.1 μg as compared with 4 μg fed as a supplement to the other group. Considering only those animals

surviving until the end of the experiment, the average cobalt content of the livers of the cobalt-supplemented rabbits was 0.140 μg/gm while the cobalt content of the other animals was 0.014 μg/gm of dry tissue. The livers of those animals which died during the course of the experiment contained 0.147 ug/gm for the cobalt-supplemented group and 0.029 μg cobalt/gm of dry tissue for the controls.

At autopsy the animals were carefully examined and for the most part appeared normal in the gross with the exception that the livers of nearly all of the animals of both groups were cirrhotic. This finding as well as the mortality rate and the failure of these animals to reproduce makes it clear that the diet used is not complete for these species. It may be that a deficiency of some other dietary essential was partially responsible for the failure to produce a cobalt deficiency.

Wunsch ('39), having reviewed the studies in New Zealand, states that forage from 77 pastures where trouble occurred contained on an average 0.038 p.p.m. cobalt on a dry matter basis, and Underwood and Harvey ('38) reported a comparable figure of 0.04 p.p.m. based on Australian studies. The present diet containing 0.0024 p.p.m. on a dry basis then gives an extremely low cobalt intake compared to that required by the sheep or cow and suggests that there is a species difference in the requirement of various animals for cobalt. Additional support for the idea that the rabbit requires less cobalt than the sheep or cow lies in the fact that the dried liver contained only 0.014 p.p.m. of cobalt as compared to the values of 0.06 p.p.m. and 0.02 p.p.m. in affected sheep reported by Underwood and Harvey ('38) and Askew and Dixon ('37), respectively.

Marston ('39) in a review article mentions a personal communication from McDonald to the effect that rabbits show no signs of cobalt deficiency when fed entirely on pasture from affected areas, and according to Marston the same is true for the horse. Working with the rat, Underwood and Elvehjem ('38) found the requirement to be less than 0.6 μg, and Houk et al. ('46), 0.03 μg per animal per day.

There is thus a growing body of evidence that cobalt deficiency is limited to the ruminants and more particularly to sheep and cattle. Some years ago Filmer ('33) suggested that some organic factor is lacking in cobalt deficiency, on the basis of their work showing that liver is effective in curing "Coast Disease," the effectiveness not being accounted for by the liver ash.

More recently McCance and Widdowson ('44) cite a personal communication from C. J. Martin that sheep suffering from "Coast Disease," while cured by cobalt given by mouth, are not cured by cobalt when given by injection. They suggest further that the element probably acts upon some of the organisms in the rumen. We suggest, further, that the microorganisms affected are those concerned with the synthesis of B-vitamins. Many of the symptoms of cobalt deficiency are comparable to those found in B-vitamin deficiency. Anorexia, for example, is a symptom of both cobalt and thiamine deficiency. Hypochromic microcytic anemia and impaired reproduction are common symptoms in both cobalt and pyridoxine deficiency.

SUMMARY

Rabbits fed whole milk and corn grain require less than 0.1 μg of cobalt per animal per day. This finding is discussed in the light of the hypothesis that the need for cobalt is peculiar to ruminant species and may be concerned primarily with biological processes in the rumen.

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The Mead Johnson and Company Award for Research on the Vitamin B Complex was presented to *Dr. William J. Darby,* Associate Professor of Biochemistry, Vanderbilt University School of Medicine, *Dr. Paul L. Day,* Professor of Physiological Chemistry, University of Arkansas School of Medicine, and *Dr. E. L. R. Stokstad,* Research Chemist, Lederle Laboratories, for their discoveries and investigations in the field of the B-complex vitamins with special reference to their studies on the chemical nature and nutritional significance of folic acid. The presentation was made at the annual dinner of the American Institute of Nutrition on Monday evening, May 19, in Chicago.

The Borden Award for 1947, administered by the American Institute of Nutrition, was presented to *Dr. L. A. Maynard* for many years of noteworthy services in the field of general nutrition and for many significant contributions to the knowledge of the physiology of milk secretion, of factors affecting milk production and milk composition, and of the requirements of animals for dietary fats. The presentation was made at the annual dinner of the American Institute of Nutrition on Monday, May 19, in Chicago.